COMMISSION ON IMMUNIZATION

Annual Report to the Armed Forces Epidemiological Board

1967 - 1968

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ARMED FORCES EPIDEMIOLOGICAL BOARD

COMMISSION ON IMMUNIZATION

DIRECTOR'S SUMMARY REPORT - FALL 1968

Since the last meeting of the Commission on Immunization, technical meetings of the AFEB commissions have been recycled so they are held in the Fall of the year. Accordingly, during the Spring of 1968, an Executive Session only was held on March 28; the first technical meeting in 18 months was held on 17 to 19 October 1968 at the Walter Reed Army Institute of Research. The first day of the meeting was devoted to a symposium on plague, cosponsored by the Commission on Epidemiological Survey. The following day and a half were devoted to a review of Commission-sponsored research and of the present state of the art in the areas of interest to the Commission.

I. Attendance	October 17	18	19
Members			
Dr. Elmer L. Becker	x	x	x
Dr. Abram S. Benenson	x	x	x
Dr. Geoffrey Edsall	x	x	x
Dr. Herman N. Eisen			
Dr. C. Henry Kempe	x	x	
Dr. Irwin H. Lepow	x	x	x
Dr. Karl F. Meyer	x	x	x
Dr. Jonathan W. Uhr	x	x	x
Associate Members			
Dr. K. Frank Austen		x	x
Dr. Edgar E. Baker	x	x	
Dr. Albert H. Coons		x	
Dr. Ross L. Gauld	x		
Dr. Thomas J. Gill III	x	x	x
Col. Wm. S. Gochenour, Jr.			
Dr. Karl Habel	x	x	x
Dr. Richard B. Hornick		x	x
Dr. George B. Mackaness			
LTC John D. Marshall, Jr.	x	x	x
Dr. J. Donald Millar	x	x	
Dr. Robert O. Oseasohn	x	x	x
Dr. Abraham G. Osler	x	x	
Dr. Alwin M. Pappenheimer, Jr.		x	
Dr. Margaret Pittman	x	x	x
Dr. Otto J. Plescia		x	
Dr. Elvio H. Sadun			
Dr. Charles V. Seastone	x	x	x

Associate Members (continued)	October 17	18	19
Dr. Willard F. Verwey	x	x	x
Dr. John C. Wagner	x	x	x
Col. Bradley W. Prior, USAF (MC), Exec. Secty., AFEB	x	x	x
Army			
Col. Ralph C. Singer, Prev. Med. Div.,OTSG	x	x	x
Col. Lyman P. Frick, HQ, WRAIR	x	x	
LTC Francis C. Cadigan, Johns Hopkins Univ.	x	x	x
LTC Robert T. Cutting, HQ, USA Med. R&D Comd.	x	x	x
LTC D. Cavanaugh, WRAIR	x		
CPT R. Edelman, HQ, USA Med. R&D Comd.	x	x	x
CPT Seymour H. White, USA Med. R&D Comd.	x	x	
Col. Herschel E. Griffin, OTSG			x
Col. Edward L. Buescher, WRAIR			x
Navy			
Capt. R.O. Peckinpaugh, NAMRU-4, Great Lakes,Ill.	x	x	
Capt. C.H. Miller, Prev. Med. Div., BUMED	x	x	x
Cdr. P.R.D. Van Peenen, NAMRI, Bethesda, Md.	x		
Capt. J.R. Kingston, ONR	x	x	x
Lt. J.M. Sachs, BUMED	x		x
Capt. L.F. Miller, BUMED		x	
Air Force			
Col. Stanley Lutz, Jr., USAF (MC), HQ, USAF, OTSG	x		
Lt. Col. Paul Nugent, HQ, USAF (AFMSPAP)		x	x
Guests			
Dr. D.M. Angevine, Assoc. Dir. Res., AFIP	x	x	
Dr. B. Cvjetanovic, WHO, Geneva	x		
Dr. Jack P. Poland, Chief, Zoonoses Section, CDC, Ft. Collins, Colo.	x	x	x
Dr. Werner A. Janssen, Asst. Chief Immunology Branch, Ft. Detrick, Md.	x	x	
Dr. Michael J. Surgalla, Chief Immunology Branch, Ft. Detrick, Md.	x	x	
Dr. William D. Lawton, Chief Microbial Genetics Branch, Ft. Detrick, Md.	x		
Dr. John H. Hughes, D/Chf. Foreign Quar., NCDC, Atlanta, Georgia	x		
Capt. Wm. H. Habig, USAMU, Ft. Detrick, Md.	x		
Col. Stefano Vivona, WRAIR	x	x	x
Dr. J.P. Lowenthal, WRAIR	x	x	x
Dr. S. Berman, WRAIR	x	x	x
C.O. Roberts, WRAIR	x		
D.N. Harrison, WRAIR	x		
Dr. M.S. Artenstein, WRAIR	x	x	
Dr. James W. Mosley, FQP, NCDC, PHS	x	x	x
Col. Dan Crozier, USAMU, Ft. Detrick, Md.	x		x

Guests (continued)	October 17	18	19
Dr. Adam J. Rapalski, Prof. Assoc., NAS-NRC	x		
I.J. Green, US Naval Biol. Lab.	x	x	x
L.F. Pierce, NAMRU-4, Great Lakes, Ill.	x	x	
E.D. Beasley, Ft. Detrick, Md.	x	x	
Johnnie M. Albizo, Ft. Detrick, Md.	x	x	
Dr. T.E. Woodward, Univ. of Md. School of Medicine	x		
Capt. S.A. Britten, MC, USN	x		
Dr. H. Bruce Dull, Med. Dir. PHS, NCDC, Atlanta, Ga.		x	x
Lt. Col. J.D. Bartley, WRAIR		x	x
H.B. Marsk, Surg. PHS, CDC, For. Quarantine		x	x

COMMISSION ON IMMUNIZATION

ARMED FORCES EPIDEMIOLOGICAL BOARD

FALL MEETING - 1968

Conference Room 341
Walter Reed Army Institute of Research
Washington, D.C.

Agenda

October 17, 1968 - 0845

Opening Remarks

SYMPOSIUM ON PLAGUE

Epidemiology

Plague in Viet Nam - Lt. Col. Cavanaugh (Washington, D.C.) and Lt. Col. Marshall (Washington, D.C.)

Field Studies of the USPHS - Dr. Poland (Fort Collins, Colorado)

Global Pattern of Plague - Dr. Cvjetanovic (WHO, Geneva)

Discussion

Clinical Aspects

Clinical - Lt. Col. Cadigan (Baltimore, Maryland)

Laboratory Diagnosis & Treatment - Lt. Col. Marshall (Washington, D.C.)

Discussion

Pathophysiology

Monkey Pathology - Dr. Meyer (San Francisco, California)

Electron Microscopic Studies - Dr. Hopps (Washington, D.C.)

Discussion

Etiological Factors

Antigenic & Virulence Factors - Dr. Surgalla (Fort Detrick, Maryland)

Genetic Studies - Dr. Lawton (Fort Detrick, Maryland)

Problems with Attenuated Strains Ev 76 - Dr. Meyer

Discussion

Protective Factors - Mr. Janssen (Fort Detrick, Maryland)
Group Discussion

Vaccination

Present Status of Killed Vaccine - Dr. Meyer

Reactions to Killed Vaccine - Col. Lutz (Washington, D.C.)

Discussion

Present Status of Attenuated Vaccine - Dr. Meyer

Logistical Problems of Attenuated Vaccines - Dr. Lowenthal (Washington, D.C.)

Discussion

Miscellaneous Comments - Dr. Meyer

Social Session, Georgian Motel, Silver Spring, honoring Dr. K.F. Meyer

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October 18, 1968 -0900

Presentations by Representatives of Armed Services

Research and Development Command - Captain Edelman
Army - Colonel Singer
Navy - Captain L. C. Miller
Air Force - Lt. Colonel Nugent

Delayed vs. Regular Immunization Schedules

Mr. Pierce - NAMRU 4 (Great Lakes, Illinois)
Lt. Col. Cadigan (Baltimore, Maryland)
Discussion

Summary Reviews of Research Sponsored by Commission on Immunization

Dr. Baker - Immunologic Studies of Schistosomiasis

Dr. Pappenheimer - Studies on the Mode of Action of Diphtheria Toxin

Dr. Edsall - Factors Influencing the Pattern of the Immune Response

Dr. Verwey - Responses of Humans to Cholera Vaccine Immunization

Dr. Uhr - Antibody Formation

Dr. Osler - Studies on the Mechanisms of Hypersensitivity Phenomena

Discussion

October 19, 1968 - 0900

Review of Areas of Concern of Commission on Immunization

Typhoid Fever - Volunteer Studies - Dr. Hornick
Reactions to Intradermal AKD - Col. Buescher

Smallpox - CV 1 - Dr. Kempe
Eradication Program - Dr. J. D. Millar
Multiple Puncture, MonoVacc - Dr. Benenson

Tet-Dip - Drs. Edsall and Pittman

Rabies - Dr. Habel

Cholera - Dr. Oseasohn

Yellow Fever - Dr. Wagner

Adjuvants - Dr. Pittman

General Discussion of Immunization Problems

II. Plague Symposium

This symposium was justified by the presence of an epidemic of plague in Vietnam with twenty-five thousand cases unofficially reported during the first eight months of 1968. The main vector of the disease, Xenopsylla cheopis, has been shown by test to be resistant to DDT throughout much of the country. Infection is derived from sylvatic animals, the incidence of disease highest in the first quarter, being inverse to the rainfall. Despite the high incidence in the civilian population, only eight cases have occurred among U.S. military personnel over the last seven years. Clinical studies have shown that the organisms persist in the body for long periods of time, in spite of antibiotic therapy; that inapparent infections are quite frequent; and that therapy, if initiated early and continued for at least seven days, is effective. Within the United States, sylvatic disease has spread eastward, with an epizootic among Eastern fox squirrels in Denver, Colorado in June, 1968. Review of the global pattern indicated the potential for plague epidemics in many parts of the world, including Venezuela, Ecuador, Peru and Brazil in South America, West Africa, East Africa, South Africa and Madagascar, Middle East to Mongolia and Nepal and Central India, Burma and Java.

Bacteriological diagnosis has proven to be effective, even with the use of transport media, involving intervals from patient to the laboratory of 24 hrs to 90 days. The use of plates containing antifraction 1 in the agar described by Mr. Albizo, which detects colonies containing fraction 1 of Congo red agar plates which detect the colonies which develop pigmentation on hemin agar, described by Dr. Janssen, permit the easy recognition of colonies with two of the five bacterial factors associated with virulence. By the use of these two techniques, virulent organisms can be selected out of a background of many contaminants, or rare mutants selected for research investigations.

Electron microscopic studies of tissue from infected monkeys revealed the deposition of intravascular fibrin and tremendous damage to the epithelialcells of the proximal convoluted tubules. Less severe damage is present in the glomerulus. The changes consist of vesiculation of the endothelial cytoplasm.

Definition of these virulence factors have been of major interest from the point of view of specific immunizing antigens, and for the selection of avirulent mutants which could be used for immunization. Classical virulent organisms have been shown to have the capsular antigen Fraction I (F 1), synthesize VW antigen (VW), form pigmented colonies on hemin agar (P), synthesize purine (Pu), and synthesize pesticinogeny (Pg), a factor related to coagulation and fibrinolytic activities.

The EV 76 strain, which has been widely used as an attenuated immunizing strain, lacks the P factor, but field data indicate that it is protective, particularly after a second dose has been given. Its use has been associated with a high reaction rate resulting in vaccination refusals. Percutaneous administration has been used in the USSR. Commission-supported studies carried out by Dr. Meyer, using the Saigon strain on human volunteers, were disappointing because of the lack of immunological response and because the recommended dose administered subcutaneously produced reactions which were considered unsuitable for mass inoculation of military personnel. Monkey studies revealed that langurs were protected against fatal but not local infection; Cerocopithecus aethiops monkeys from Ethiopia were found to respond similarly to the langurs. No monkeys immunized with the Saigon strain ever died. This strain is uracil-requiring and as such is noninvasive and nonpersistant. In the attempt to increase its antigenicity for man, an EV 76 strain, obtained from Madagascar in 1951, was passed through 5 iron-sulphate-treated guinea pigs. The resultant isolate, designated EV 76 51f, passed the tests of safety defined by Girard for attenuated plague strains, but when given to green monkeys of Ethiopian origin, the animals became sick and some died of plague septicemia. Cercopithecus monkeys coming from Kenya developed positive blood cultures and died of disease after as few as 2,180 organisms were administered subcutaneously, with a pathological picture equivalent to that induced by a virulent strain of plague.

The M-23 strain isolated by Dr. Burrows and his coworkers in Porton, England, is not encapsulated and, therefore, devoid of Fraction I. Animal studies carried out by the Ft. Detrick workers on rhesus monkeys have shown that this strain is as infectious as the standard virulent plague bacillus, producing a local lesion at the site of intradermal injection, and/or regional lymphadenopathy with no evidence of systemic disease and no deaths. With these lesions a precipitin antibody demonstrable by gel diffusion appears as well as a positive C reactive protein test. The latter correlated excellently with the ability of these monkeys to resist an aerosol challenge with 10^5 P. pestis one month after inoculation. Whereas the virulent P. pestis strain was lethal at aerosol doses of 1800 or above, none of 38 monkeys challenged with 5×10^5 aerosol doses of the M-23 strain succumbed, but aerosol doses of 1000 or more uniformly elicited a positive C reactive protein and immunity to aerosol challenge. (Aerosol doses of 8×10^4 or less of EV 76 failed to infect rhesus monkeys). It is evident that this is a strain of promise which must be investigated in the Kenya monkeys and perhaps in man.

Present protection against plague thus depends on killed vaccine. The occurrence in military personnel in Vietnam of only eight cases of plague over the duration of American involvement there, while at least 65 cases of murine typhus were confirmed during the year June '67 to '68 is taken as suggestive evidence that the vaccine affords effective protection but no studies have been performed showing that fleas are as frequently infected with P. pestis as with Rickettsia mooseri. Comparing the protection afforded by an aqueous E medium vaccine, with an alum adjuvanted aliquot with comparable numbers of organisms in langurs at a 1 cc. dose, revealed that a single inoculation of either vaccine elicited HA antibodies but poor mouse protection indices (MPI), a 0.2 ml. booster dose was given 86 days later and 40 days afterward 66% of monkeys were protected against plague challenge. Comparable results were obtained in man; a single inoculation of vaccine reduced the MPI below 10 in only 16%; below 5, in only 4% of volunteers. (In monkey studies, no langurs with an MPI above 10 survived challenge, only those with an MPI below 5 resisted relatively large challenge doses). A booster inoculation of 0.2 ml. brought the MPI to below 10 in 65% and below 5 in 26% of the human subjects. However, the alum adsorbed preparation, while several-fold greater more antigenic in the standard mouse potency test than the aqueous product, proved unstable since, when used after 9 months of storage, a 1 cc. dose produced an MPI below 10 in 20% of volunteers; none had an MPI below 5.

Prior studies had indicated that alum adsorption results in acceptable mouse protective indices 3 to 4 times more frequently than found after aqueous suspensions; this storage deterioration obviates the use of this formulation. The present aqueous USP plague vaccine (E medium), followed by a booster of 0.2 ml. at 60 days, stimulates protective antibodies adequate to confer relative immunity against fatal bubonic plague in about 65% of human subjects. Of practical importance, reactions to the current vaccine are minimal. Primary doses in the Air Force recruit reception stations have been given with jet injection, with the reaction rate reported from Lackland and from Amarillo AFB range between 0.02 and 0.03%.

In review, our present status is one in which the second dose of killed vaccine is necessary for effective vaccination and that while hopefully this could be improved by the use of alum adjuvant, evaluated formulations have been unstable on storage. We have greater expectations from an attenuated live vaccine. However, past experience in Indonesia and in Madagascar suggests that immunity with this too became effective after the second dose. Present preparations, protective doses, are associated with severe reactions. There is hope that the M-23 strain, which retains its original infectivity but not its virulence, might offer greater promise and this must be put through the appropriate testing procedures. The concern with use of attenuated vaccines is that of the development of aberrant strains, which, while safe in test animals, might be virulent for man. Data available indicates that the freeze-dried, currently available strains retain their viability for two to four weeks at 22°, 41 weeks at 4°, and two years at -20°.

The symposium crystalized many thoughts and provided an opportunity for three separate working groups to compare their information and to plan appropriate protocols for accelerated future progress. It was climaxed by an evening dinner session honoring K. F. Meyer for his tremendous contributions to the military over the long years he has explored the techniques of plague prevention and control, seeking those most effective for military use; and for his fundamental contributions to our understanding of the pathogenesis of plague. Pertinent comments were made by Dr. Stanhope Bayne-Jones, bearing on K.F. as a scientist, by Colonel H.E. Griffin, MC, Chief, Preventive Medicine Division, Office of The Surgeon General, Department of the Army, on his contributions to the military; by Dr. T.E. Woodward, on his contributions to medicine, and Colonel W.S. Gochenour, VC, on his contributions to veterinary science. K.F. reviewed for us his career and we relived his original isolation of encephalitis virus; as usual, we are amazed at how young a great man of 84 can be. Although he threatens to take a well-earned holiday, we hope he will continue to work with us for many years more as we improve our control over plague.

III. Commission-Sponsored Research

The annual progress reports of Commission-sponsored contracts are appended to this report. Highlights of progress and of the discussion at the meeting are presented here.

a. Dr. E. E. Baker (Immunologic Studies of Schistosomiasis)

reported his efforts to separate the active fractions of extracts of adult Schistosoma mansoni. This project has been hampered by scarcity of the adult worms with which to work, and the formation of a precipitate in initially clear preparations, associated with loss of serological activity. Biological activity was followed only by agar gel precipitation; best results were obtained by extraction with Tes buffer (N-Tris (hydroxymethyl) Methyl-2-amino ethane sulfonic acid) at pH 7.2, which resulted in a clear extract containing five antigens, stable for at least one month in the frozen state, or at 4° C. and which reconstituted completely after lyophilization. Attempts to separate these antigens by column filtration on Sephadex, Sepharose 4B, or on DEAE cellulose failed. This project is being terminated because of the difficulty in procuring sufficient material for these studies and because it has not been possible to follow the separation by skin activity, one of the ultimate objectives of this contrast.

b. Dr. G. Edsall (Factors Influencing the Pattern of Immune Response) reported on the continuing observations on four groups of women in Colombia who were given one injection of a low or high dose of tetanus toxoid (5 or 25 Lf), with a low or high amount of an aluminum phosphate adjuvant (0.77 or 3.86 mg/dose). At 28 days the response to the high-high (toxoid-adjuvant) dose was clearly superior to that of any other combination, but the levels of the high-low and low-high groups were comparable. However, a year after a second injection of the same material, the mean geometric antitoxin level was comparable in both groups receiving the high dose of toxoid but, more important, all who were tested a year after the second dose, had protective levels of antitoxin, except one person in the high-high group! Of particular interest, however, was the observation that the antitoxin levels 28 days after the high-high dose fell into two distinct distribution groups. In one group, antitoxin levels ranged between less than .0025 and .04; the second group ranged between .08 and 1.2 units (levels in those with seven-day responses, which were excluded, ranged between 0.64 and 40 units). Since this was also observed among men at Ft. Detrick, this could not be attributed to endocrine levels. Because of the frequency with which a single high-high dose evoked protective levels of circulating antitoxin, the group will be followed for a year to observe the adequacy of a single dose for long-term immunity. Other significant observations included the fact that the human response to these four products correlated well with the WHO assay performed on guinea pigs, but not on mice. Also, in some of these Colombian women, low levels of antibodies were suggested by hemagglutination but this could not be corroborated by toxin neutralization tested in animals; a similar observation has now been made in residents of the Boston area. The nature of this cross reactivity has not been resolved as yet.

c. Dr. H. Eisen (Antibody Structure and Function), who is presently working at Oxford, England, was unable to attend the meeting. His report covers the continuation of his work on the basic aspects of antibody structure and the immunization process. He has shown that para proteins from humans and mice with multiple myeloma not only are physico-chemically and antigenically like antibodies, but have antibody activity against simple well defined chemical structures. Since these proteins are generally much more homogeneous than antibodies obtained following artifical stimulation, this brings closer the possibility of determining the exact structure of the site of the antibody which combines with antigen.

d. Dr. K. F. Meyer (Studies on the Immunization of Man Against Plague) reported his studies interspersed through the plague symposium and are discussed thereunder.

e. Dr. A. G. Osler (Studies on the Mechanisms of Hypersensitivity Phenomena) reported studies carried out with Major Carter L. Diggs on the humoral immunity to rodent malaria. They very clearly showed that the serum of rats which had recovered from a bout with Plasmodium berghei contains a humoral antibody which, when passively administered, suppresses or prevents infection with this malarial parasite. The evidence indicates that it acts on the merozoite while this is free in the blood stream, traveling to the new host red cell. This observation raises hope for a hyperimmune gamma globulin for the treatment of critically ill malaria patients. This outcome must await considerable confirmatory studies on primates.

Dr. Osler's studies have shown that the classical histamine release from rabbit platelets by antigen-antibody combinations can occur without fixation of the antibody to the platelet, that the histamine release is associated with cell damage as indicated by an increased rubidium efflux rate (equivalent to potassium), that the rubidium or histamine-release requires the presence of a single divalent cation, Mg^{++}, and that the rates of release vary directly with antibody concentration and only occur in antibody excess. By contrast, leucocytes obtained from the human allergic donor release histamine when exposed to the appropriate antigen with no damage to the cell, as evidenced by absence of enhanced potassium or rubidium loss. The potency of pollen extracts can be assayed with these cells with greater reproducibility and sensitivity than by skin test. Blocking antibody in human serum can be measured during the hay fever season, and the severity of illness can be followed by the amount of pollen antigen required to release 50% of the histamine from the cell. Peripheral leucocytes from nonallergic donors can be made sensitive by incubation with a serum of ragweed sensitive donors, (the immunoglobulin fixes to the cell before histamine release can occur), permitting characterization and quantitative estimation of human reagenic antibodies, providing a test tube substitute for the Prausnitz-Kustner reaction. The ability of the ragweed patient's serum to sensitize leucocytes varies somewhat during the course of the year, being highest immediately following the ragweed season; lowest in the summer months. The variation was such that the postseason titer of 12 patients averaged three times that of the preseason titers. These studies provide us with an in vitro system for developing therapeutic techniques and therapeutic controls, without the hazard of hepatitis transmission.

f. Dr. A. M. Pappenheimer, Jr. (Studies on the Mode of Diphtheria Toxin) gave the terminal report on this ten-year project. These studies disclosed that diphtheria toxin inhibits protein synthesis by preventing the action of transferase II; this enzyme moves the growing peptide chain along the ribosome so that it can accept the next amino acid as it is built up into a protein molecule. The action of this enzyme depends on nicotinamide adenine dinucleotide (NAD); the toxin acts on NAD with liberation of free nicotinamide and the ADP-ribose moiety attachs at, or near, the active site of the transferase molecule. Working with tissue culture cells, he was able to demonstrate that only about 25-50 toxin molecules attached to each cell; they remain at the surface, never actually getting into the cell itself, but are sufficiently deep within the cell wall that they cannot be affected by trypsin or by antitoxin. When the toxin is mixed with antitoxin, this attachment to the cell does not occur, a situation resembling that seen when mouse cells are used (mice are resistant to diphtheria toxin). The toxin action is enzymatic; the 25-50 molecules on the cell completely inactivate the transferase II within the cell in 20 minutes, and there are assumed to be about 10^7 transferase II molecules per cell. No method has been discovered for neutralizing the effect of the toxin once it is within the cell; but extracts can be restored to normal aminoacyl transfer activity by adding small amounts of toxin together with excess nicotinamide.

g. Dr. J. W. Uhr (Antibody Formation) has continued his work on the nature of antibody formation, with particular interest in carbohydrate incorporation, and release of the formed antibody from the cell. He has also been studying ways of continuously removing specific antibodies from the circulation of animals by extracorporeal circulation of the blood through a specific immunoabsorbent; such an approach may be of great usefulness in the treatment of human allergic reactions which involve the continuing reaction of circulating antibody. He has recently turned his attention to the production of persistent local streptococcal infections in baboons with the hope of developing experimental models which are closer to human rheumatic fever and poststreptococcal glomerulonephritis than any available heretofore. At least one animal showed definite glomerulonephritis and one a severe myocarditis with focal muscle necrosis. None showed findings typical of rheumatic fever but the experiments are continuing.

h. Dr. W. F. Verwey (Responses of Humans to Cholera Vaccine Immunization) immunized three groups of volunteers with either the CRL cholera vaccine (which had been used in the Pakistan-SEATO Cholera Research Laboratory field studies of 1963 and 1964), commerical cholera vaccine (CV), and the commercial vaccine diluted 1:10 (DCV). He compared the responses to a single dose or two dose schedule, either one week or one month apart, and also the response to a booster at six months. The vibriocidal antibody was followed in bleedings taken 1, 2, 3, and 6 weeks after each immunizing dose. The studies indicated that the best group response followed initial immunization and not reimmunization. For initial immunization, 2 doses (0.5 and 1.0 ml) a week apart was preferable; the second day evoked an antibody response in some who otherwise would not have responded. Grossly increased quantities of antigen (mouse Inaba antigenicity of CRL was 20 fold that of CV) produced only slightly improved results. Indeed, imbalance in the relative amounts of several antigens (CRL: Inaba reference ratio was 25.0; CRL: Ogawa reference ratio 3.6) may result in serious depression of antibody response; the anti-Ogawa response to CRL was actually poorer than that to DCV, although CRL had 20 times more Ogawa mouse antigenicity!

Reimmunization at a 4-week or 6-month interval did not produce a booster effect; it produced peak antibody titers that were no higher and frequently significantly lower than those following initial immunization. Small dose initial immunization, followed by a standard dose booster, did not improve the reimmunization response, and a double dose reimmunization with a one-week interval did not improve the situation. A poor response to initial immunization was correlated with a better response to the booster. The only beneficial effect of a 6-month booster dose was found in a higher mean vibriocidal antibody level remaining at the end of a 6-month period. After a single dose of CRL vaccine, the antibody level reached a peak, declined during the first 6 months, and remained stable at an elevated level for the rest of a year; for this full period of observation, the bulk of vibriocidal activity was in the gamma M fraction of serum.

It is evident that much additional work must still be done to understand the phenomena involved in immunization with relatively complex antigens such as the bivalent cholera vaccine, and to define the most protective immunization formulation, dosage and schedule.

IV. Review of Areas of Concern of the Commission

a. Acute Respiratory Disease

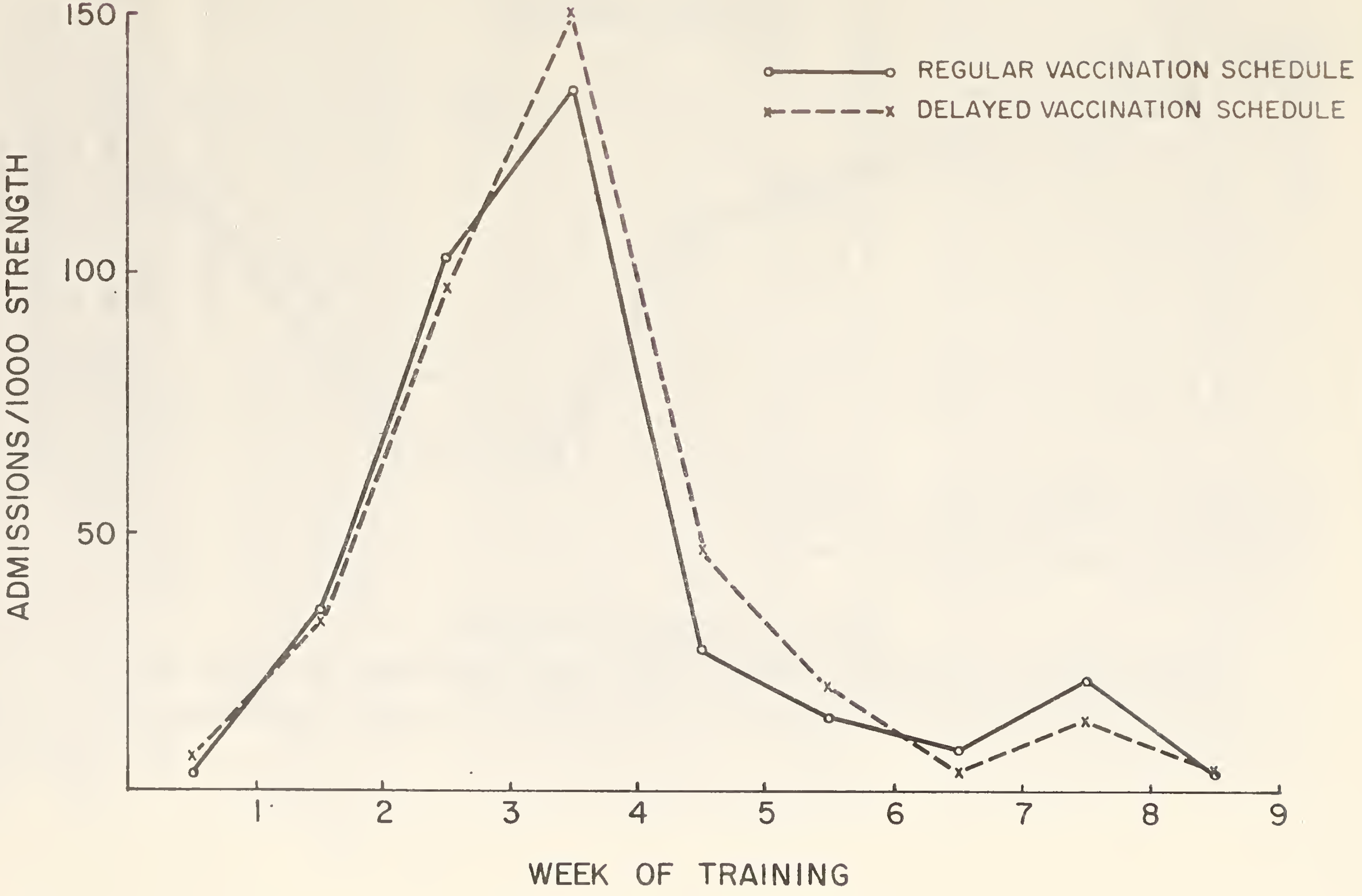
For several years, the Commission on Immunization has discussed the studies which had been carried out by the Navy at Great Lakes and by the Army at Ft. Dix, on the effect of immunization on respiratory disease rates. Because of the significance of these two studies on military immunization programs, it was felt essential to assemble all the information for presentation and discussion. Mr. Pierce presented the Great Lakes data; his report is appended hereto. In essence, whole training companies received the regular immunization schedule in force in the Navy while others were given only the polio and influenza vaccines, (adenovirus during the season of prevalence). All other antigens (TAB, T-d, and smallpox) were delayed until the second half of the training schedule. This study was carried out from 1 January 1961 to 30 June 1962. Approximately 38,000 men received the regular vaccination and 27,000 the delayed schedule. There was approximately 20% less respiratory disease among those in the delayed schedule companies than those on regular immunization. Analyzing the experience on a cohort basis, i.e., comparing the ARD incidence in the companies who started training at the same time, this reduction, while small, was consistent throughout the study period. Since those on the delayed schedule were not given a placebo, the question arose whether infection was a consequence of attending dispensaries for the purpose of immunization. To clarify this issue, 312 men in the delayed schedule were found who had visited sick call five or more times for nonfebrile illnesses, such as blisters; 31 or 10.9% developed ARD in contrast with 65 cases in 461 matched controls (14%) who had not visited the sick call; the difference is not significant.

Colonel Cadigan presented the Ft. Dix results in which the input of one week in January, 1962 was studied. This covered five companies totaling approximately 1200 men, who were randomized within their companies on the bais of the last digit of their serial number. This was a double blind study with placebos given to those who did not receive active antigens; all antigens were delayed until the second half of the training period in those receiving placebos. Forty-seven percent received routine immunization and 43% the delayed schedule. Four hundred thirty-nine men were hospitalized for ARD with the peak in the third week. Sixty percent of these illnesses were attributed to adenovirus, mostly Type 4 but some 7's. There was no difference in experience in ARD incidence of the two groups (see page 15). There were 567 dispensary visits not resulting in hospitalization, with no significant difference between the groups (see page 15). Twenty-six cases of pneumonia occurred in this group; 12 in the routine and 15 in the delayed group. Thus, in this study the time when antigens were given had no effect on the incidence of disease.

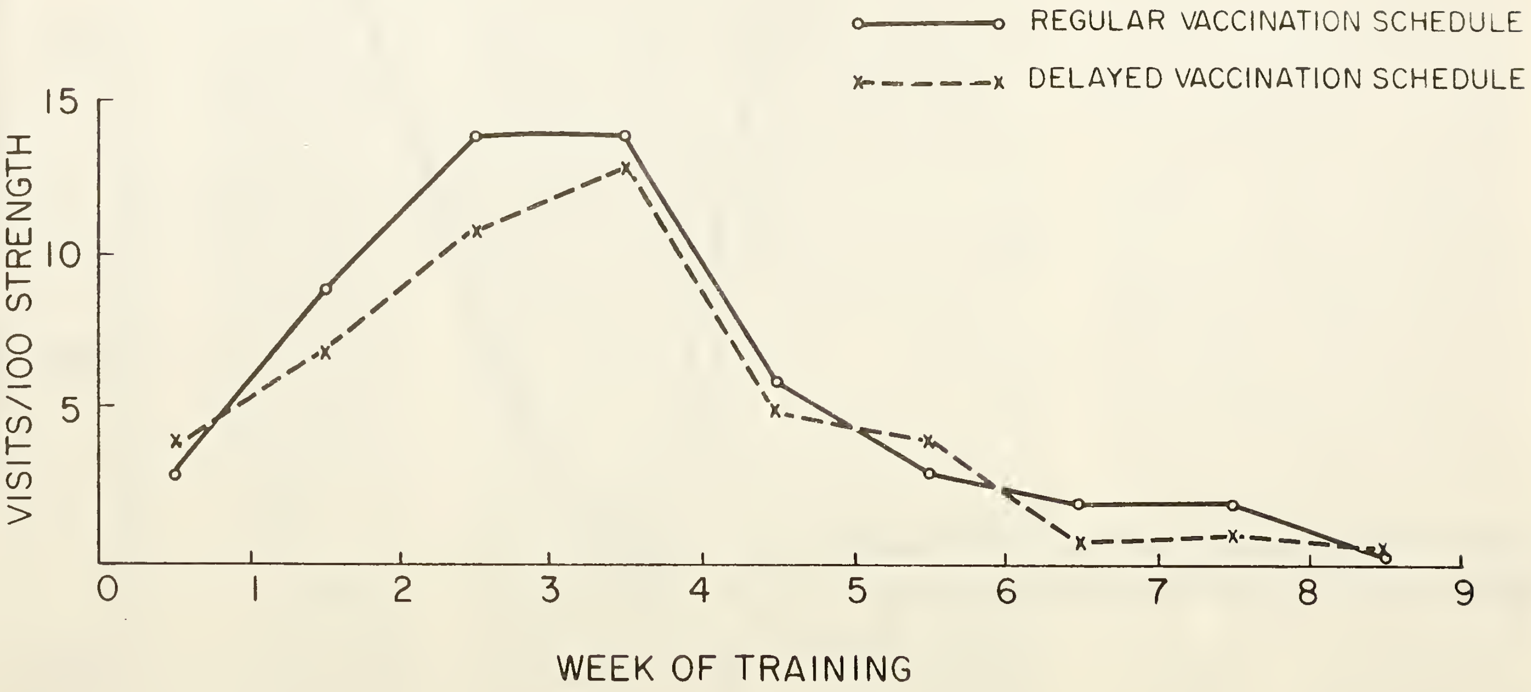
In the discussion, it was brought out that there were a few significant differences between these two studies. At Great Lakes, TAB vaccine was being given during the second and third week of training, a period when the incidence of ARD infections is increasing sharply; furthermore, this was given in three doses of TAB for a total of 2 ml. while the Army gave three doses, for a total of 1.5 ml. At Ft. Dix, no immunizations were given to any men during weeks 2 and 3, while at Great Lakes TAB was given each of these weeks during the period when infection was increasing. In a second point of considerable potential importance, was the difference in design, in that, with a relatively small difference in attack rate (20%), the alternation of men within a unit might conceal an effect which would be evident when the complete unit was exposed to the same stressful situation. Finally there would appear to be epidemiological differences between Great Lakes and Ft. Dix since, at Great Lakes, throughout the year there is a consistent pattern of respiratory disease, with the peak incidence at the third week of training. At Ft. Dix, on the other hand, this typical pattern is not seen until late January or early February, continuing through February, March and April. At other times of the year, the peak tends to occur toward later in the training cycle.

In discussion, it was concluded that sufficient information was not available, in view of the suggested differences in epidemiological patterns, to make a solid recommendation bearing on military practice. However, the Great Lakes experience could be meaningful, and indicates that if feasible, an added antigen should not be given at the time when infection is actively occuring within a command.

ADMISSIONS TO HOSPITAL FOR ARD FROM FIVE STUDY COMPANIES, BY WEEK OF TRAINING AND VACCINATION SCHEDULE—FORT DIX, 1962.



DISPENSARY VISITS FOR ARD (Excluding those hospitalized on day of visit), BY WEEK OF TRAINING AND VACCINATION SCHEDULE - FIVE STUDY COMPANIES, FORT DIX, 1962.



b. Typhoid Fever

(1) Dr. Hornick summarized the studies with which he has been involved at the University of Maryland. Volunteers were given two different oral typhoid vaccines: one (Typhoral) was prepared by the Bayer Company, containing 33 billion Ty 58 organisms, plus para A and para B; the other, (Taboral) contains 100 billion Ty 2 organisms. In doses recommended by the manufacturers, they evoked shortlived antibody rises against the O, H and Vi antigens in a low percentage of the volunteers. An oral challenge of 10^5 typhoid organisms, which had been an ID_{25} in previous studies, was given 8 to 10 weeks after the oral administration. In the conditions of this study, however, this proved to be an ID_{50} challenge and no significant protection was demonstrated; however, it must be recalled that, at an ID_{50} level, no protective effect could be demonstrated with parenteral vaccines.

The Maryland group has also been studying the comparative virulence of different organism strains. S. typhi Ty 2, which has been a laboratory strain for 47 years, had an ID_{50} of 3×10^6, in comparison with the Quarle's strain, a recent Maryland isolate, which had an ID_{50} of 2.8×10^7. Strains lacking Vi antigen (Ty 2-W and 0901) also produced disease in man at 1.9 and 3.1×10^8 dose levels, but the disease seemed to be somewhat less severe clinically and was more difficult to diagnose, since these strains were much more difficult to recover. Had the infecting organism not been known, many of these challenged cases could have been considered FUO. At a 7-log challenge dose, 26% developed disease with nonVi, non-motile strain, 0901, in comparison with 51% developing disease after challenge with the Vi-containing organisms. A Vi-positive streptomycin-dependent strain, administered together with oral streptomycin for a week, could be recovered from the stool for 9 days, but produced no symptoms and evoked no antibody response; a comparable dose subcutaneously, produced a prompt serological response.

(2) Colonel Buescher reported the experience with the AKD vaccine given intradermally. All of 12 volunteers developed an area of erythema associated with pain; two had systemic reactions; one developed some slough at the site of immunization. A study at Ft. Bragg, administering heat-phenol and AKD vaccine by jet gun, showed no difference in reaction rates or severity. These studies have been repeated at Great Lakes, using the lot of AKD which is ready for delivery to the military, with the same results.

c. Hypersensitivity Phenomena after Vaccination - Dr. Kempe reported his experiences with children developing an atypical pattern of measles five to six years after vaccination with KKK or KKL measles vaccines. These cases have high fever, and a marked rash resembling rickettsial rash more than anything else. They may have hemorrhagic manifestations, pneumonia, pleural and peritoneal exudate, and are ill for two to three weeks. The diagnosis is made by recognizing association with typical measles cases. They did not have cough or Koplik's spots. The Academy of Pediatrics recommends that any child who has had killed measles vaccine receive a dose of live vaccine. This may result in a very sore arm eight days later in about 50%, with perhaps a one-day fever, but the serious atypical disease is avoided. Colonel Singer advised that killed measles vaccine had never been introduced in the military supply system. However, it could have been purchased locally, or been given to dependents by civilian physicians. Accordingly, based on an AFEB recommendation, the Services asked that dependent immunization registers be scanned and those who had received KKK or KKL measles vaccine be given a dose of live attenuated vaccine.

A second instance of vaccine-induced hypersensitivty reported by Dr. Kempe was noted in the children receiving respiratory syncytial virus vaccine. This had been given in a controlled study and involved about 100 military dependents in Denver, at Fort Ord and in Washington. This vaccine proved to be highly sensitizing and actually potentiated clinical disease. The group receiving this vaccine experienced 15-fold increase in frequency, and the severity of RS disease was also increased so that, in some, it was associated with massive pneumonia. This sensitization continued into a second year, so that some children who had severe RS disease in the first season after the vaccine was given, developed serious pneumonia the second year. The parents of these children have been advised of the potential problem.

d. Smallpox Vaccination

(1) Dr. Kempe reported his experience with 2,000 eczematous children vaccinated with the attenuated vaccinia virus, CV 1-78. Detailed analysis was only available on the first thousand patients; 326 were vaccinated by the multiple pressure technique; the others by the subcutaneous injection of 1,000 TCID$_{50}$ or 3,000 TCID.$_{50}$ The multiple pressure vaccination produced a small attenuated take and no eczema vaccinatum cases occurred. Subcutaneous injections with a tuberculin syringe or jet injector produced redness or swelling on the eighth to tenth day. Ninty percent of the CV 1 vaccinated children remained afebrile in comparison with 55% of healthy children vaccinated with standard Dry-Vax by multiple pressure. In 23% of those vaccinated with CV 1 by multiple pressure, there was an antibody response but no local lesion. The geometric mean neutralizing titer of eczematous children receiving 3,000 TCID$_{50}$ subcutaneously or by multiple pressure vaccination was comparable to that of normal children vaccinated with Dry-Vax percutaneously. The height of fever and the severity of illness did not corrolate with the antibody level.

(2) Dr. Millar discussed the National-Communicable-Disease-Center-supervised eradication program in 19 countries of West and Central Africa, which is based on mass vaccination with intradermal jet injection. To date, 58 million vaccinations have been performed in 310 million people. This has resulted in a marked drop in the incidence of smallpox, so much better than anticipated that the second phase of eradication, i.e., a search and destroy operation for each focal outbreak, has been instituted in all but two countries. This operation is basically an African effort. The American contribution has been 44 technicians out of the 2700 people involved, payment of American salaries, procurement of jet guns, vehicles, and vaccines. Vaccination of newborns is being combined with other vaccination campaigns. The basic program itself is combined with measles vaccination, a disease which hits 99% of African children by age 4 and kills 10% of these.

(3) Dr. Benenson reported on the use of the multiple puncture rather than multiple pressure technique of percutaneous vaccination, using the bifurcated needle. In the multiple puncture technique, the needle is held perpendicular to the arm, rather than parallel; for bifurcation of the needle, it is broadened so that perforation of the skin is unlikely, if at all possible. Since this technique may be simpler to master, its efficacy was evaluated on three groups of approximately 150 recruits at Ft. Dix. All men were vaccinated on the left arm by standard multiple pressure, using 15 to 20 pressures of the bifurcated needle. One group was vaccinated on the right arm with 5 punctures, the second with 10 punctures, and the third with 15 punctures, through a drop of vaccine deposited on the skin from the slot in the needle. One week later the reactions were read and recorded with the following results.

	Number of Punctures		
Method	5 (149 men)	10 (144 men)	15 (150 men)
Multiple Pressure	96%	88%	85%
Multiple Puncture	79	89	90
Either	97	95	96

Percent of Vaccinees with Major Reactions After Vaccination by Multiple Pressure (15-20) or Multiple Puncture Method

These results indicate that 10 punctures are as effective in eliciting major reactions as the standard multiple pressure technique with 15 to 20 pressures; and that 15 punctures yields a higher percent of major reactions than a comparable number of multiple pressures. The discomfort associated with the multiple punctures was apparently no greater nor more unpleasant than that with multiple pressures; if the heel of the hand is fixed on the skin, so that the punctures are essentially at the same point, small scars can be anticipated.

(4) Dr. Benenson also discussed the problem posed by the inclusion of the MonoVacc plastic vaccinator on the supply table. Earlier studies, and the manufacturer's advertising, indicate that it may be acceptable for primary vaccination only; however, its inclusion on the standard supply list involves the danger that it will be used for revaccinations. This is not justified by any data which has so far been presented. An evaluation in collaboration with the manufacturer is proposed.

e. Tetanus-Diphtheria Immunization

Dr. Pittman discussed the problem posed by using the adult type tet-dip for primary immunization against diphtheria. Originally it was included to serve as a booster to earlier established immunity.

However, it is being used now for primary immunization, with no information of its effectiveness. Dr. Edsall discussed the requirement for a new formulation with a reduced tetanus component for adult use, in view of the fact that approximately 75% of young adults now have been immunized. Hopefully, this material would have sufficient antigenicity that it could elicit primary immunization, and he feels this could be done with a proper formulation containing only 2 to 5Lf of tetanus toxoid. It was pointed out that the military situation requires a high titer of antitetanic immunity while diphtheria has not been a practical problem for many years. Concern was expressed at the lack of requirement for a high level of purity of the tetanus component. It was felt that if the military specifies 1500 Lf per milligram of nitrogen, the manufacturers could meet this requirement.

f. Rabies

(1) Dr. Habel discussed the present status of rabies immunization. The combination of a series of vaccine doses and a single dose of antiserum continues to be the recommended postexposure treatment for man; and booster doses of vaccine continue to be recommended when antiserum has been given. Human antiserum can be obtained with gamma globulin levels as high as that of horse, and animal studies indicate that when a high titer, highly potent vaccine is used, the interference with homologous serum is no different from that observed with antiserum from the heterologous species. However, there is still concern that with a vaccine of relatively low potency (but still passing minimum requirements) this interference phenomenon might become important. Unfortunately, the present duck embryo vaccine may fall into this category; however, there is no evidence based on failure rates in recent years, to indicate that the duck embryo vaccine is any less effective than brain tissue vaccines in protecting those who have been bitten by a rabid dog, even though it is less potent by any type of quantitative potency test done in animals. Work in several different laboratories promises an improved vaccine, either from a tissue culture source purified and concentrated by zinc sulfate precipitation and sucrose gradient fractionation, or derived from brain material but purified by column chromotography, or by concentrating the viral content of the present preparation.

(2) Pre-exposure immunization is still highly recommended. It is desirable that successful immunization be confirmed by establishing the presence of antibodies. When this has not been done, it was agreed on subsequent exposure they should be treated as individuals who have not had pre-exposure immunization. If they had been shown serologically to be positive, the reduced schedule recommended by WHO is sufficient. Antiserum has been shown to interfere with the booster response; therefore, if there has been any evidence of neutralizing antibody, the booster doses of vaccine are indicated but antiserum locally would be contraindicated.

g. Cholera

Dr. Oseasohn discussed the findings obtained by Dr. Mosley and his group in Dacca, indicating that in the absence of suspected cholera, it is possible to have a high incidence of serological conversions, particularly in young individuals, and then an excellent correlation exists between infection rates and vibriocidal titer. The problem of the carrier state and the fact that immunization does not affect this was discussed at moderate length, particularly from the point of view of quarantine and the inclusion of cholera among the quarantinable diseases. Dr. Oseasohn raised the question of immunity to the toxic factor reported by Craig before this Commission a few years ago. In the course of acute cholera, patients develop antitoxic immunity; however, children, at the time of their acute attack, may have appreciable levels of toxin-neutralizing potency. Dr. Benenson pointed out that young children in Pakistan have toxin-neutralizing potency in their serum which disappears by age 15, while vibriocidal antibody increases with age, so that at about age 7, 50% were positive; at age 10 about 85% had vibriocidal titers.

h. Yellow Fever

Dr. Wagner reported that the NIH has succeeded in removing the contaminating avian leucocysis virus from the 17 D vaccine strain, so that there is now available an attenuated yellow fever strain of 17 D virus at the same level of passage as the present vaccine. The avian leucocysis-free new seed is identified as 17 D-51-A; it has a titer of 10^6 to 10^7 intracerebral mouse LD_{50} per ml. The separation of viruses was achieved by filtration. The new strain has been tested in 200 monkeys in comparison with the standard strain, and the antibody responses were comparable in the two groups. Two hundred prison volunteers have been tested; followup difficulties were encountered but the evidence available indicates no difference in behavior of the new strain. There is now available 25 to 50,000 doses ready for clinical trials; there are three smaller lots of 5,000 doses each in production. These clinical trials are essential before standard routine use. It was felt that the military immunization would be a logical place for such studies.

Dr. Mosley raised the question of the instability of the vaccine, pointing out the difficulty encountered with the present requirement that all doses be administered immediately after the vaccine is reconstituted. It was agreed that there was justification for maintaining rigid requirements with this vaccine which, to date, has been so effective in performance, but that information on stability of the freeze-dried vaccine in storage, and of the reconstituted vaccine available, is desirable.

i. Adjuvants

Dr. Pittman reported on the studies being carried out under contract to the Division of Biologics Standards on adjuvants. A large study is being carried out to answer the question of carcinogenesis; mineral oil, peanut oil and all ingredients that go into the mixtures are under test. The metabolic fate of the ingredients is being studied in rats and squirrel monkeys. Alum monostearate is one of the factors under study; however, bacterial antigens have not been included. These studies are expected to replace our ignorance of the long-term effects of adjuvants and whether or not their use is associated with the hazard of undesirable delayed reactions. The results of these studies are expected to become available within the next year or two.

j. Miscellaneous

(1) Two technical bulletins have been prepared; the one on cholera is under final review for submission. The one on immunization still has two chapters to be completed. The remainder has been circulated and suggestions integrated.

(2) Colonel Singer discussed the Army position with regard to immunization. Because of the publicity when one man refused immunization this past year, a DA circular was published, reaffirming the policy of forcible immunization; i.e., using the minimum force necessary to administer it. In general, it is only necessary to touch the man and most religious groups accept this as evidence of force. This satisfies their conscience and then they permit routine immunization. He asked that the current circular be included in the proposed TB Med.

V. Acknowledgements

The Director wishes to express his appreciation to the members of the Commission for their cooperation and support. Appreciation must also be expressed for the help provided by Captain Britten, Miss Gilbert and the staff to the new Director. We will miss Captain Britten, and take this opportunity to wish him the best in his new assignment. We welcome Colonel Prior as the new Executive Secretary.

Respectfully submitted,

Abram S. Benenson, M.D.
Director
Commission on Immunization

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The Effect of Delayed Immunizations on Acute Respiratory Disease in Naval Recruits

Special Report to the Commission on Immunizations
of the Armed Forces Epidemiological Board

Washington, D.C. 20315

by

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Great Lakes, Illinois 60088

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THE EFFECT OF DELAYED IMMUNIZATIONS ON ACUTE RESPIRATORY DISEASE IN NAVAL RECRUITS

Acute respiratory disease (ARD) is disproportionately prevalent in military recruits (1-5). The navy recruit population represents less than 5% of the entire Navy population, yet nearly 50% of all the respiratory disease in the Navy occurs among recruits (6).

Figure 1 shows the results of one of the earlier studies of the Naval Medical Research Unit No. 4 (NAMRU-4) in recruits at Great Lakes (7). Seasonal fluctuation of respiratory disease rates is evident. They are high in the winter, low in the summer, and gradually increase in the fall and early winter months. This figure also shows that there is considerable company variation in the number of recruits admitted for ARD.

One of the most striking observations made repeatedly by NAMRU-4 is that there is a great disparity between the enormous amount of respiratory disease experienced by these men within the short span of 8 to 10 weeks and the small fraction of this total illness which is represented at sick call. Longitudinal surveys conducted in the barracks indicate that only 10-20% of all respiratory disease is reported to sick call by recruits (8-9).

Longitudinal and cross-sectional surveys conducted throughout the years have revealed that most recruits experience from two to three discrete respiratory disease episodes during their training. Less than 10% of the recruits escape ARD (7, 8, 9). The results of a cross-sectional survey conducted in 25, 000 recruits during the years of 1955 and 1956 (10) indicated that 49.6% of the men in all weeks of training, regardless of the season of the year, had respiratory illness symptoms (Fig. 2). There is less febrile disease in the summer months than in the winter, but nearly 2% of men in all weeks of training had a temperature of 100° or greater when surveyed.

Recruits reporting to sick call with respiratory disease have done so with such regularity that a pattern has been established which is referred to as the fingerprint of respiratory disease in Naval recruits (10-11).

Figure 3 shows data from 1951 and indicates that the first 4 weeks of training are the periods of time in training when respiratory disease is reported most frequently, which coincided with the time period when all the recruit routine immunizations were given. This suggested that the exaggerated incidence of ARD could be due either to the immunization reactions or their superimposition on a mild respiratory disease condition. Such an effect would make a recruit more prone to seek treatment for the mild acute respiratory disease, which otherwise, may have remained unreported. It seems reasonable to assume that a man with a sore throat, cough and low grade temperature, who developed a primary smallpox reaction and was immunized that day with typhoid vaccine, might be a little more prone to seek treatment for his sore throat than he would be if he did not have this added influence.

To determine what role this immunization schedule had on reporting acute respiratory disease, a study was conducted from 1 Jan 1961 to 30 June 1962. The study population consisted of approximately 65,000 navy recruits who began training within this time. They were divided into two groups on the basis of their training company which were designated as "regular" or "delayed". Each group was placed on one of two immunization schedules as shown in Table 1. The delayed group received their typhoid-paratyphoid, diphtheria, tetanus and smallpox vaccinations in the second half of training --- 5th to 9th weeks, while the regular group was immunized as usual in the first 4 weeks of training. Standard doses of vaccines were used. The first typhoid-paratyphoid inoculations were given in 1 ml doses. Placebo injections were not given. The experimental unit was the training company composed of 65 to 90 men, all of whom were immunized either on the regular or delayed schedule. This study design was necessary since most activities in recruit training are handled on a company basis. Recruits delayed in training for 3 or more days for administrative or medical reasons must be transferred to a less advanced company. This method of study allowed reassignment to a company with an identical schedule which eliminated duplication or missing of immunizations. The Recruit Training Command training staff assigned the companies to either a regular or delayed immunization schedule prior to the commencement of training. This was not done in rotation, but from each week's complement of companies, at least one company was assigned to each immunization schedule. The seasonal respiratory disease prophylactic procedures --- influenza, adenovirus vaccines and benzathine penicillin were given to all recruits on either immunization schedule at the same time of training. The training routine of the two immunization groups was identical.

Admission records may be influenced by subjective factors and may not give a valid index of the total acute respiratory disease experience in naval recruits. Therefore, a random sample of all recruits in training was made to determine the prevalence of respiratory disease symptoms. One recruit, selected at random from each training company every two weeks was interviewed. This amounted to a 4.5% sample of all recruits in training. The randomly selected recruits were called to a clinic where a history regarding respiratory disease symptoms was obtained. A blood specimen for serology was collected at the time of the interview and again after two weeks.

Official medical records on each hospital admission, as well as outpatient visits to the infirmary, were abstracted for illness data on each recruit prior to his departure from recruit camp.

DISCUSSION OF RESULTS

A reduction in cases of respiratory disease and pneumonia requiring inpatient care was noted in recruits on the delayed immunization schedule, as shown in Figure 4. The overall relative reduction during the 18 months of this study amounted to 20.5% (12-14). Figures 5 and 6 show the distribution of hospital admissions by the week of training for each of the 12 months of the year 1961. Reductions of ARD experienced by the delayed group ranged from 14.7% to 35%. The 6 months of 1962 showed similar distrubutions. This reduction in acute respiratory disease requiring inpatient care in these recruits on the delayed immunization schedule was observed in the first 4 weeks of training. No similar increase in admitted acute respiratory disease was observed in the delayed group when their immunization program commenced. It would appear that recruits can tolerate extensive immunization later in training when the natural incidence of acute respiratory disease is less.

As previously pointed out, we were greatly concerned that hospital admission rates represent only a small proportion of the total acute respiratory disease experienced by recruits. Further, an admission must be preceded by the recruit voluntarily seeking aid for his condition. Therefore, it is possible that the differences in the hospitalized illness between the treatment groups observed may be explained as merely motivation to seek treatment when experiencing an immunization reaction in conjunction with acute respiratory disease. The data in Figure 7 shows the infirmary visits for afebrile conditions which did not result in an admission. There is no difference in the reporting of this condition by recruits on either immunization schedule. The data, however, for the febrile conditions, which were not admitted, show a small, but similar, reduction as observed with the hospitalizations (Fig. 8). It would appear that only the more severe ARD was altered by the delayed immunization schedule.

The results of the random sample of the entire recruit population is shown in Figure 9. Again, as was observed in reported acute respiratory disease, those recruits surveyed on the regular immunization schedule had respiratory disease symptoms more frequently in the first half of training than did those recruits on the delayed schedule. As was observed with hospitalization, these differences were not great, only about 20%. The slight excess of symptoms elicited in the last half of training, when the delayed group was receiving immunizations, is not statistically significant. This was a consistent pattern which was observed throughout the entire period of study. It must be pointed out that while the person making these observations did not solicit information regarding immunizations, complaints regarding an immunization reaction were often brought out during the interview. Thus, these observations were not made in a double-blind manner, since placebos were not utilized.

In the Spring of 1962, in connection with another study in this population (14, 15), 70-mm chest films were taken on approximately 2,700 fully active recruits surveyed during the 4th week of training. Thus, it was possible to determine quite objectively the effect of routine immunizations, given early in training, on the prevalence of pulmonary infiltrates. It should be pointed out that recruits, during initial processing, routinely receive a 70-mm chest X-ray and any recruit with an infiltrate is hospitalized. Thus, this population started their training without infiltrates. It can be observed, in Table 2, that there was a marked reduction in hospitalized pneumonia in those recruits who were on the delayed schedule and had not received their routine immunizations at the time of the X-ray survey. Moreover, there was still a further reduction in the prevalence of pulmonary infiltrates among recruits in active training who had not reported to sick call or had not been detected clinically. These data support the previously reported reductions in acute respiratory disease observed in hospital admissions and from random sampling. It would appear that extensive immunization programs administered during the period of time when the recruit population is experiencing the major part of its respiratory infections indeed increases the total incidence of acute respiratory disease. Further, the observed increase in illness is not simply due to immunization reactions causing increased reporting of disease.

An opportunity to observe the effect of delayed routine immunizations on adenovirus, as well as non-adenovirus-associated disease, occurred when an adenovirus vaccine evaluation study was conducted in this population in the late spring and early summer of 1962 (14). The recruits participating in the adenovirus vaccine study were randomly divided into the vaccine or placebo group and received their vaccinations prior to being formed into companies. Therefore, each company, whether on the delayed or regular immunization schedule, contained vaccinated and placebo-treated recruits. Figure 10 presents the hospital admission rates for acute respiratory disease experienced by those men assigned to the two immunization schedules who received either adenovirus vaccine or a placebo. It will be observed that there was less admitted acute respiratory disease in both the vaccinated or placebo groups noted from those recruits who were assigned to companies on the delayed immunization schedule. Thus, it would appear that the effect of reducing acute respiratory disease by delaying routine immunizations is not limited to adenovirus disease, but to acute respiratory disease of other etiology as well.

Table 3 presents the adenovirus serology of randomly selected recruits not given adenovirus vaccine. The delayed immunization group had fewer serological conversions to adenovirus, particularly in the first half of training. These differences, however, are not statistically significant. Although observed throughout the entire study, the differences on the specific adenovirus rate can only partially explain the difference in illness reported by these recruits. The specific disease admission rates for non-adenovirus was also less in the delayed group.

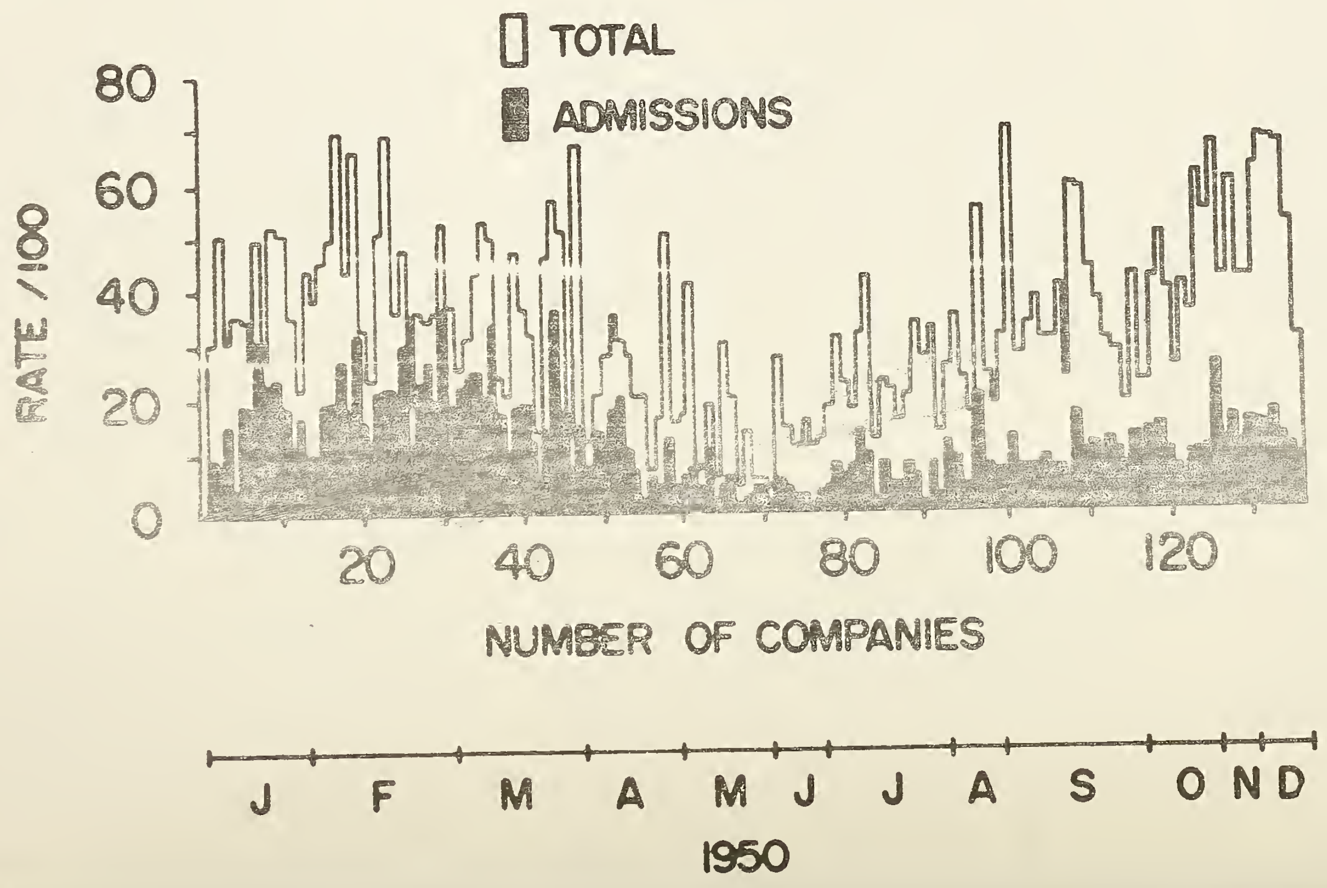
The ARD re-admission rates for recruits from both immunization schedules in the various weeks of training are shown in Figure 11. The re-admission rate is considerably higher than the admission rate for both groups. Recruits on the delayed immunization schedule, however, had a lower re-admission rate than did those on the regular schedule. It would appear that an illness either selects out a disease-prone individual or makes an individual more prone to subsequent illness. The latter hypothesis may explain the mechanisms involved with the excessive acute respiratory disease observed in those recruits who experienced the intensive immunization procedures at the time of the greatest exposure to acute respiratory disease. It may be that a slight alteration in the natural host resistance prepares the individual for a more severe acute respiratory disease, which ultimately results in hospitalization.

Another interpretation of these data would be that once an individual is hospitalized and placed into the environment of other ill people, he contracts their illness. It has been suggested that the increased illness observed from recruits on the regular immunization schedule might be due to contacts with disease made while receiving immunizations. Immunizations were given in the infirmary in an area used specifically for that purpose. In an attempt to determine the role of the medical facility in the spread of acute respiratory disease, all men from the delayed immunization companies who had reported to sick call 5 or more times for only non-acute respiratory conditions before the 5th week of training were selected. These men were matched with all other men in the same company who had not reported to sick call for any condition before the 5th week of training. A total of 312 men from the delayed schedule were found with 5 or more visits to sick call for non-respiratory disease conditions. These were matched with 461 men from the same companies who had not reported to sick call at this time in training. Thirty-four (10.9%) of 312 of those who had sought treatment for only non-respiratory disease conditions before the 5th week of training were subsequently hospitalized for ARD.

However, 65 of the 461 or 14.1% of the matched controls were subsequently admitted for ARD. This difference is not statistically significant. It is interesting that those who visited the medical facilities for non-ARD conditions early in training were hospitalized for ARD less frequently than those who had not visited the medical facilities. These data should be cautiously interpreted. Some of the men who sought treatment for non-respiratory conditions and were hospitalized for that condition may have received treatment for an accompanying acute respiratory disease at the same time. The converse has been noted frequently in vaccine studies. One observes that the vaccine group that experiences less ARD reports more non-acute respiratory disease conditions. It would appear that a certain percent of the population is going to be hospitalized at least once for some condition. Further, there is a potential penalty of being set back in training for being hospitalized.

The medical facility may be an important source of spread. However, it must be borne in mind that the navy recruits' training is somewhat different than that of the other services. The bulk of his time is spent indoors in clasrooms. The barracks, which house several training units, drill halls, swimming pools, large recreation centers, large mess halls, etc. are all places of congestion with ample opportunity for mutual exchange of flora and fauna.

INCIDENCE OF ACUTE RESPIRATORY DISEASE IN NAVAL RECRUITS DURING PERIOD OF RECRUIT TRAINING



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PER CENT OF RECRUITS WITH RESPIRATORY SYMPTOMS IN VARIOUS WEEKS OF TRAINING AND SEASONS EPIDEMIOLOGICAL SURVEY, GREAT LAKES, ILL. 1955-56

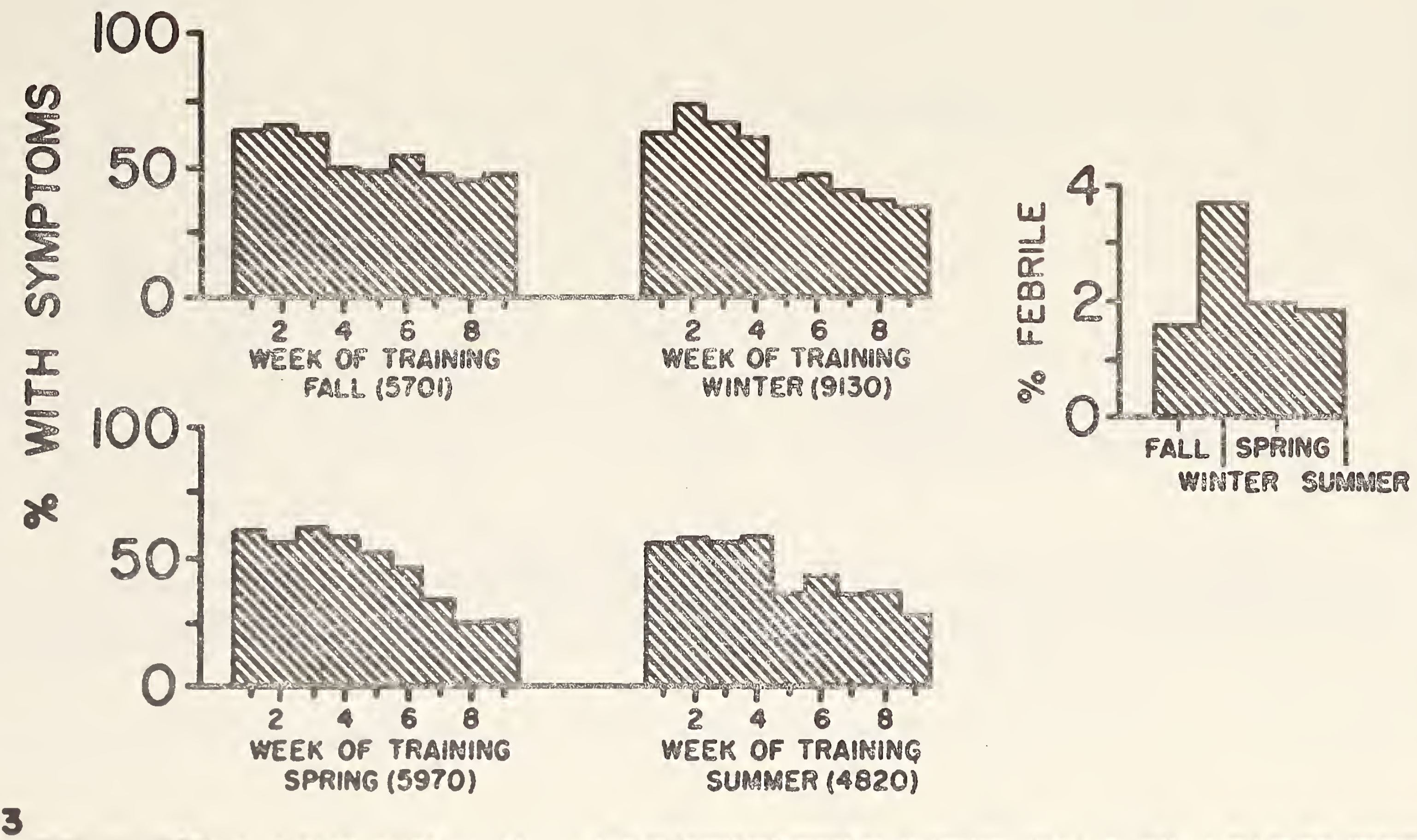


FIGURE 2

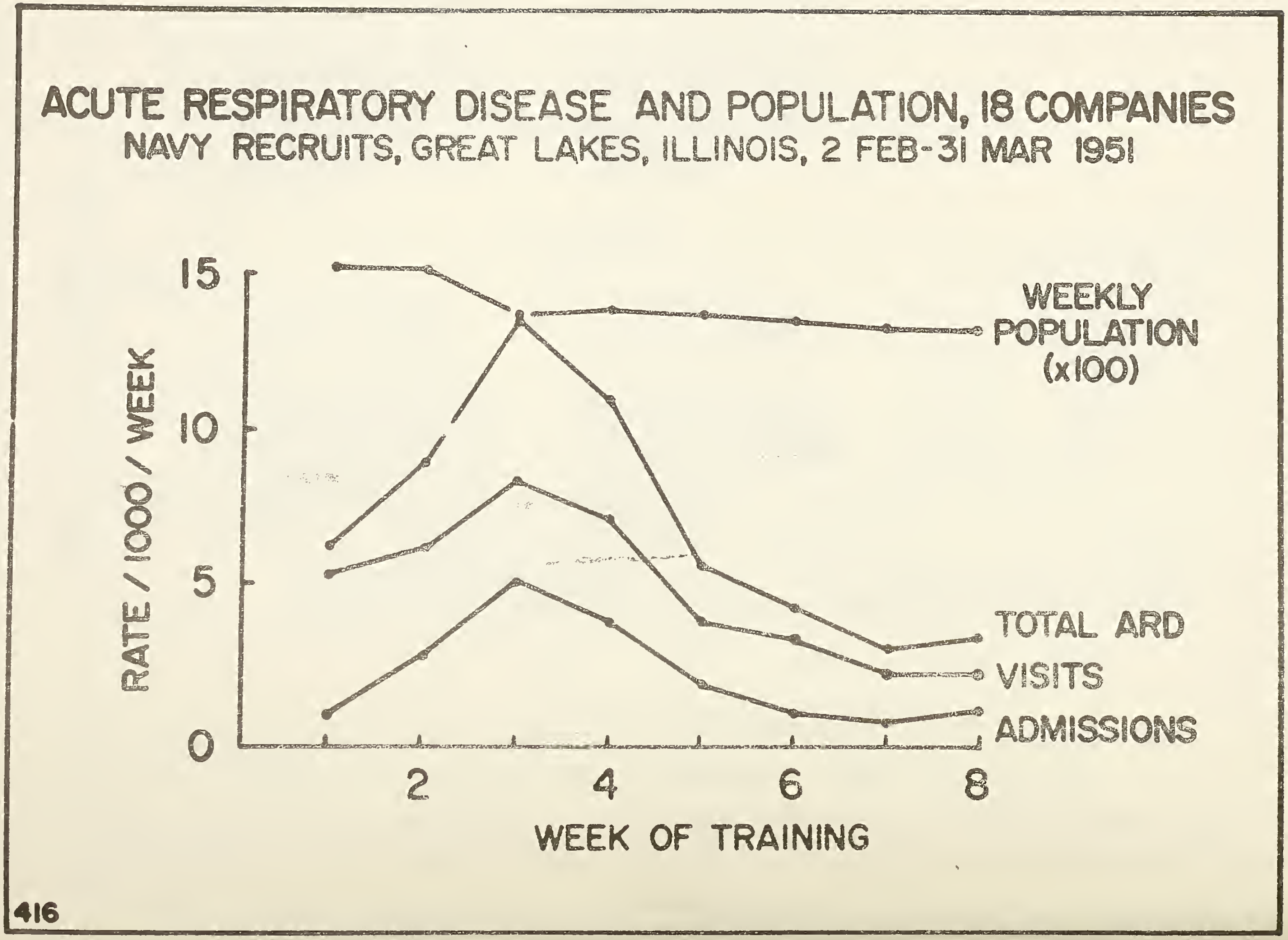

ACUTE RESPIRATORY DISEASE AND POPULATION, 18 COMPANIES
NAVY RECRUITS, GREAT LAKES, ILLINOIS, 2 FEB-31 MAR 1951
RATE / 1000 / WEEK
15
10
5
0
2
4
6
8
WEEK OF TRAINING
WEEKLY POPULATION (x100)
TOTAL ARD
VISITS
ADMISSIONS
416

FIGURE 3

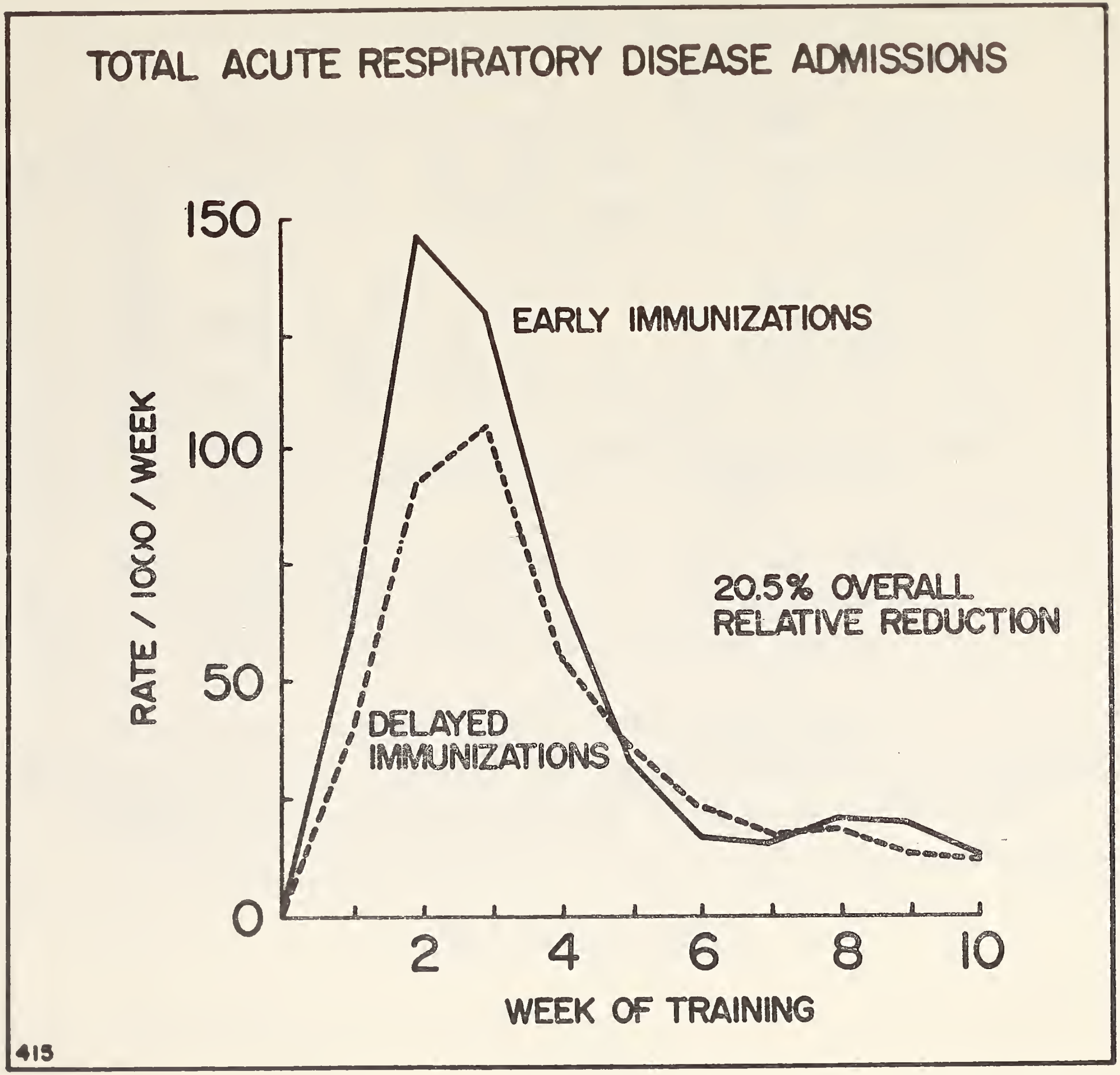
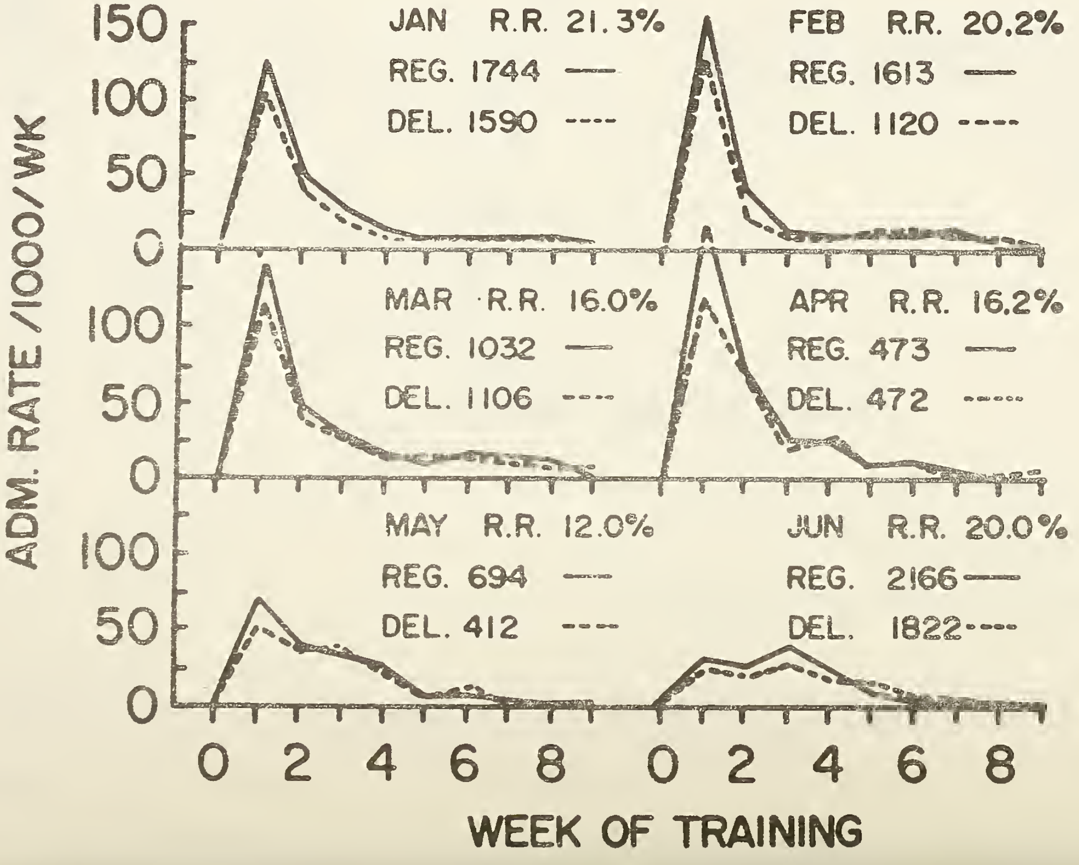

TOTAL ACUTE RESPIRATORY DISEASE ADMISSIONS
RATE / 1000 / WEEK
150
100
50
0
2
4
6
8
10
WEEK OF TRAINING
EARLY IMMUNIZATIONS
DELAYED IMMUNIZATIONS
20.5% OVERALL RELATIVE REDUCTION
415

FIGURE 4

ACUTE RESPIRATORY DISEASE IN MONTHLY COHORTS
OF RECRUITS ON REGULAR AND DELAYED IMMUNIZATION SCHEDULES
NTC GREAT LAKES, JAN-JUNE, 1961



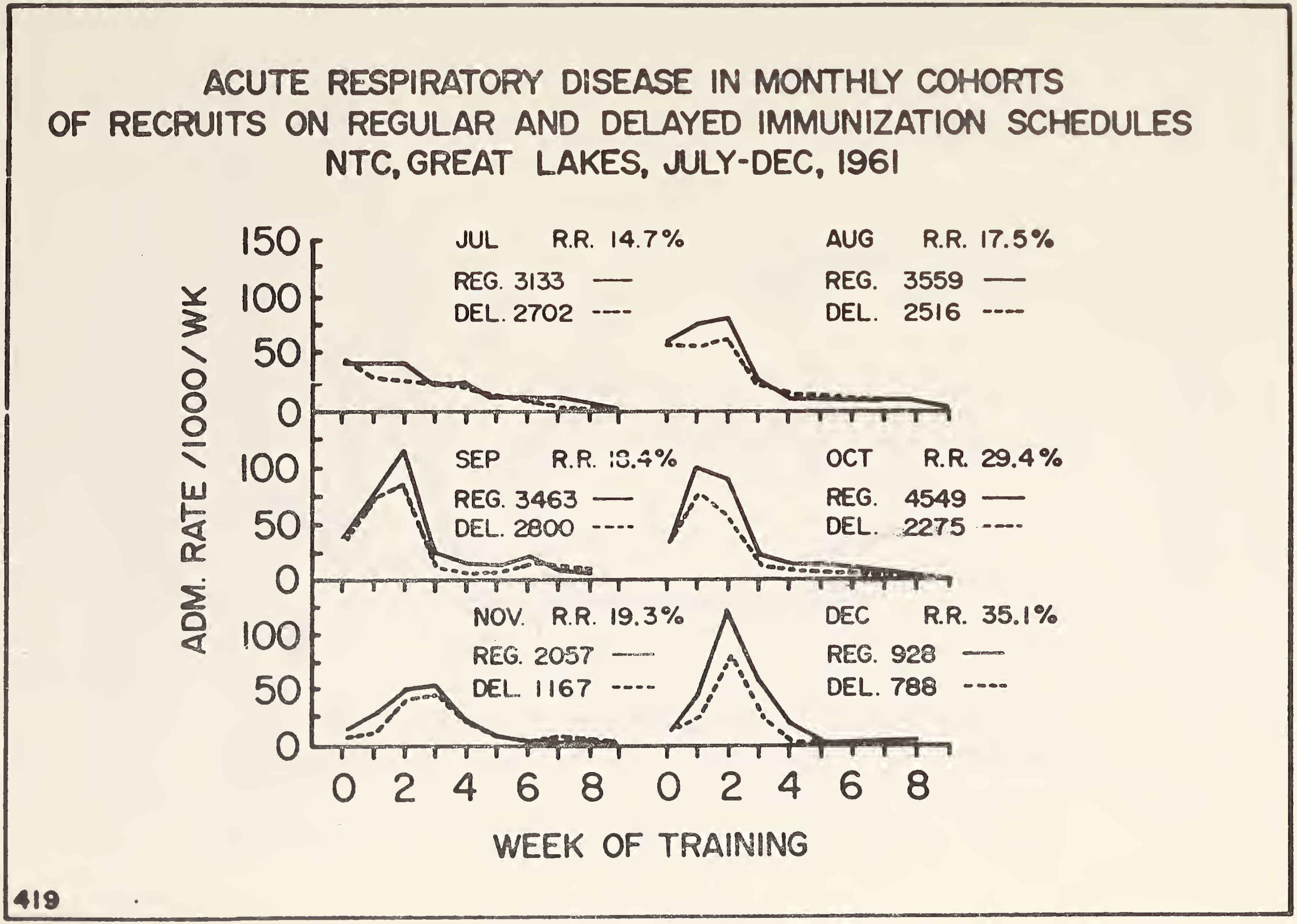
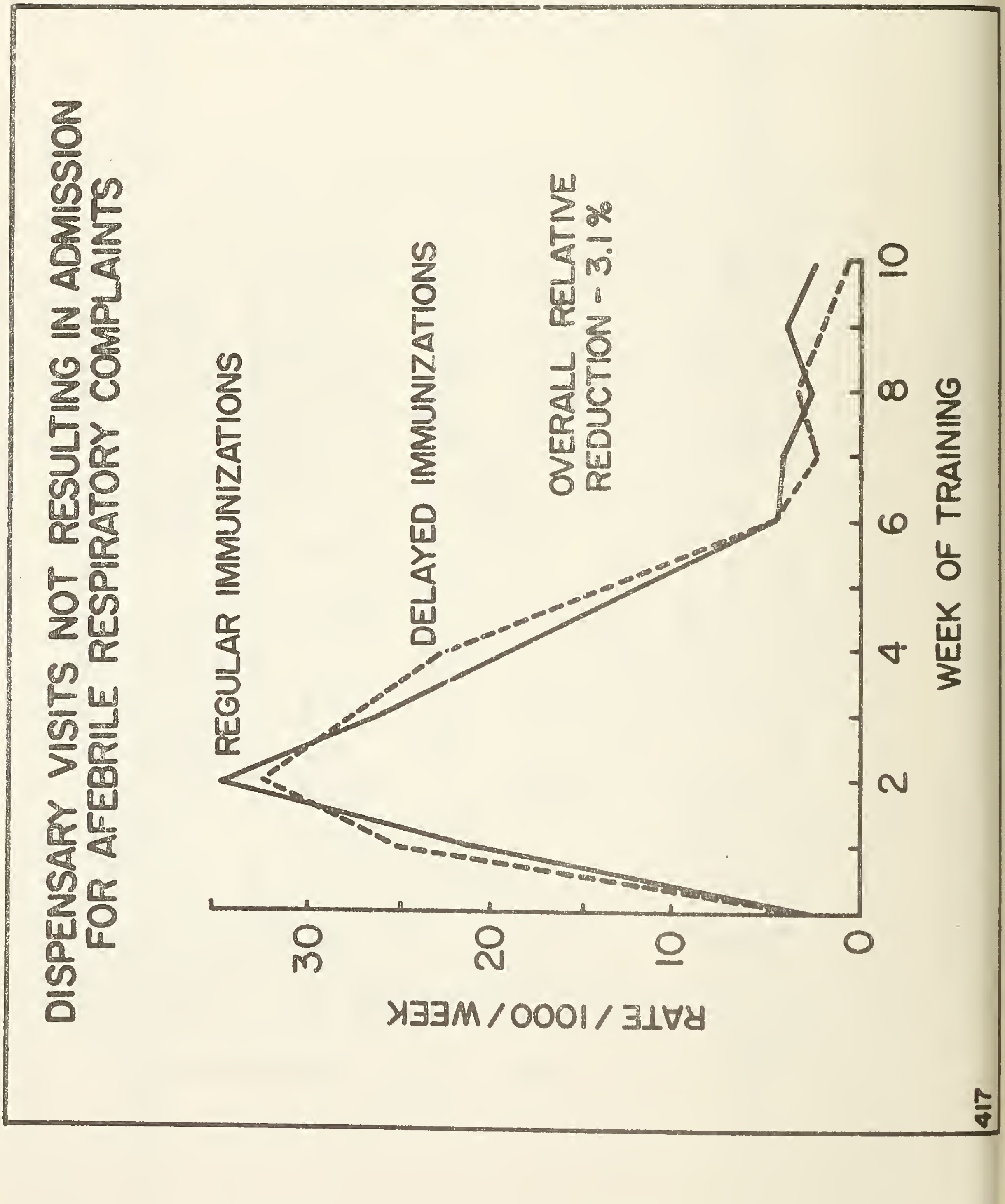

ACUTE RESPIRATORY DISEASE IN MONTHLY COHORTS
OF RECRUITS ON REGULAR AND DELAYED IMMUNIZATION SCHEDULES
NTC, GREAT LAKES, JULY-DEC, 1961
ADM. RATE /1000/WK
150
100
50
0
JUL R.R. 14.7%
REG. 3133 —
DEL. 2702 ----
AUG R.R. 17.5%
REG. 3559 —
DEL. 2516 ----
SEP R.R. 18.4%
REG. 3463 —
DEL. 2800 ----
OCT R.R. 29.4%
REG. 4549 —
DEL. 2275 ----
NOV. R.R. 19.3%
REG. 2057 —
DEL. 1167 ----
DEC R.R. 35.1%
REG. 928 —
DEL. 788 ----
0 2 4 6 8 0 2 4 6 8
WEEK OF TRAINING
419

FIGURE 6



DISPENSARY VISITS NOT RESULTING IN ADMISSION FOR AFEBRILE RESPIRATORY COMPLAINTS

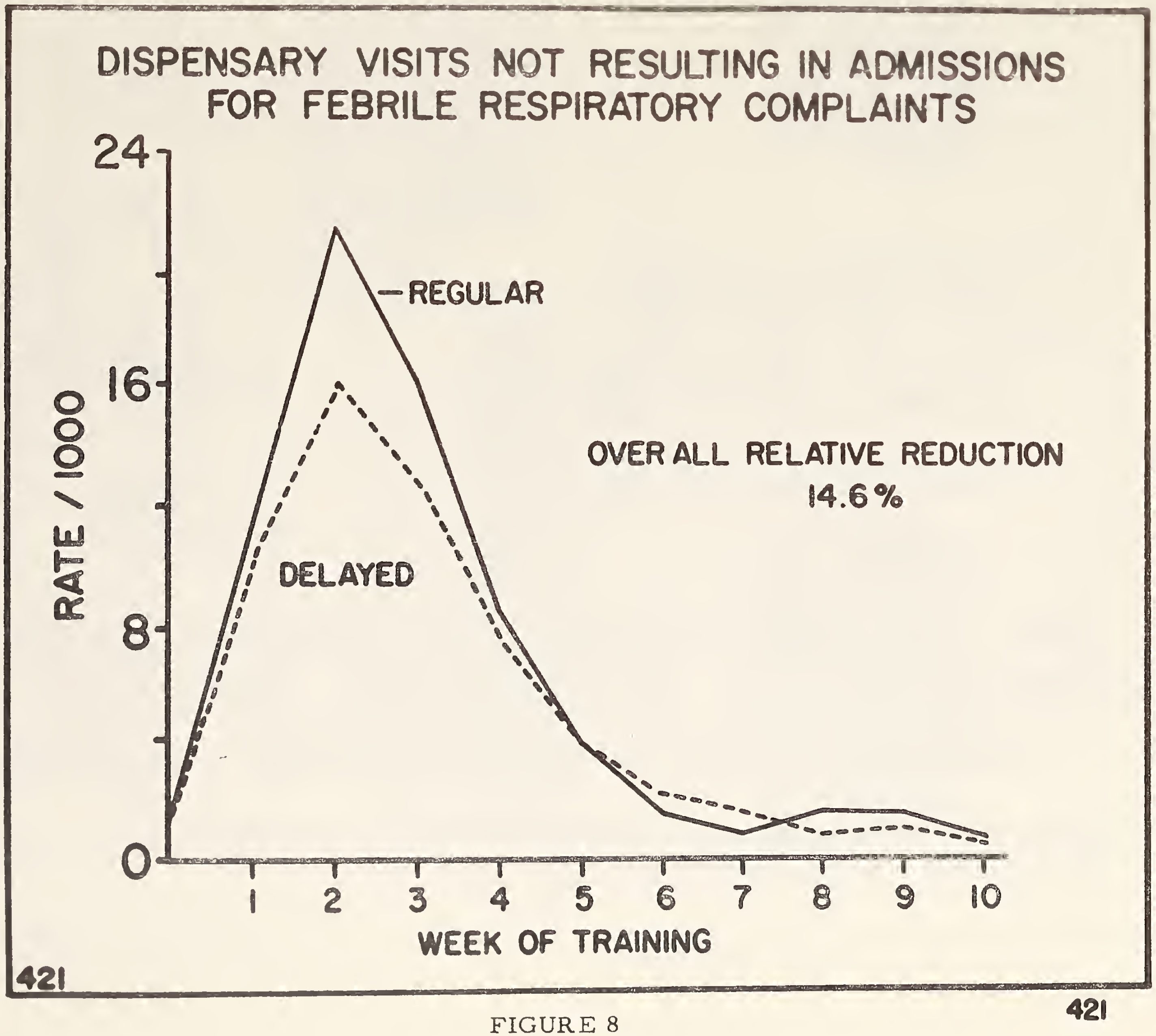
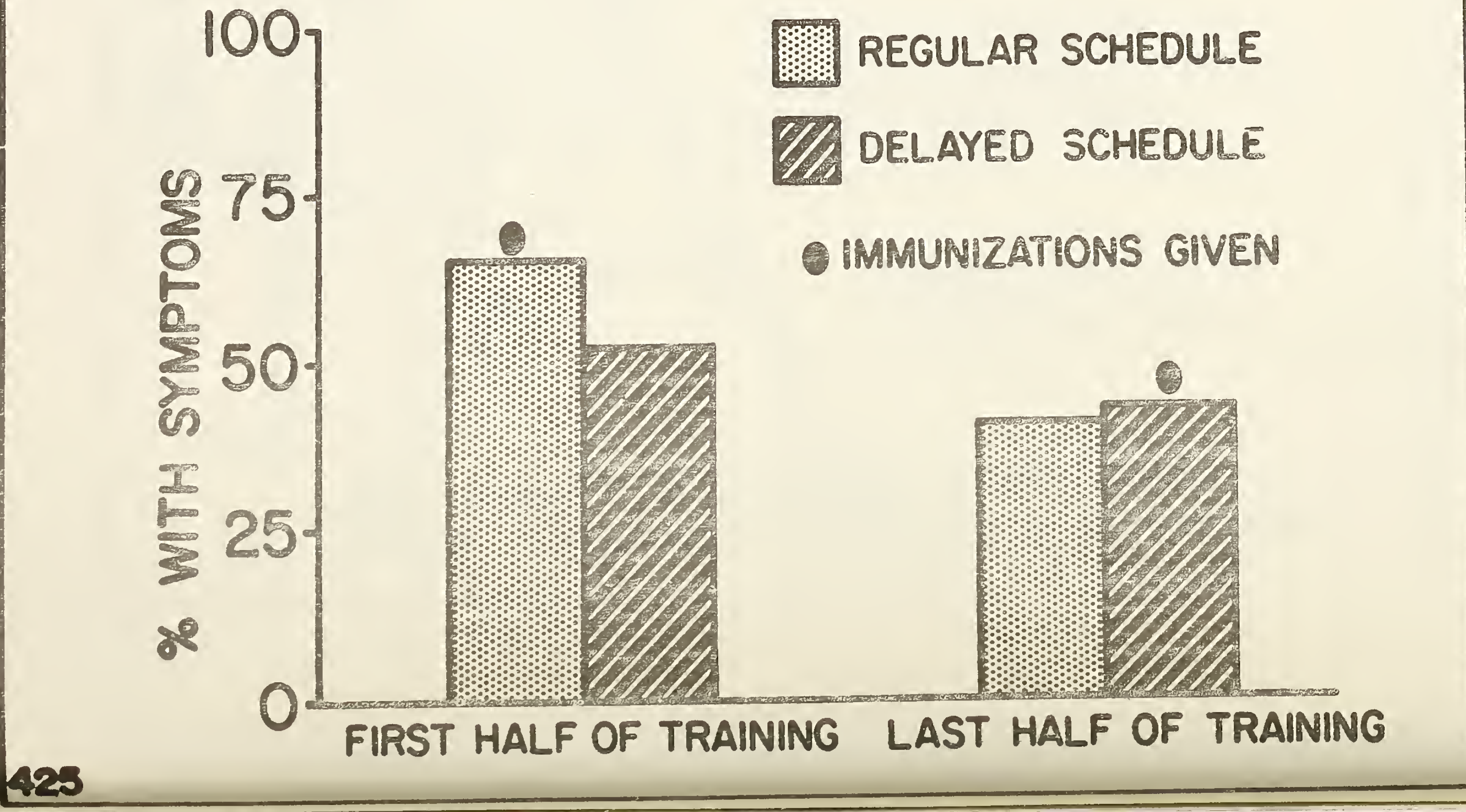
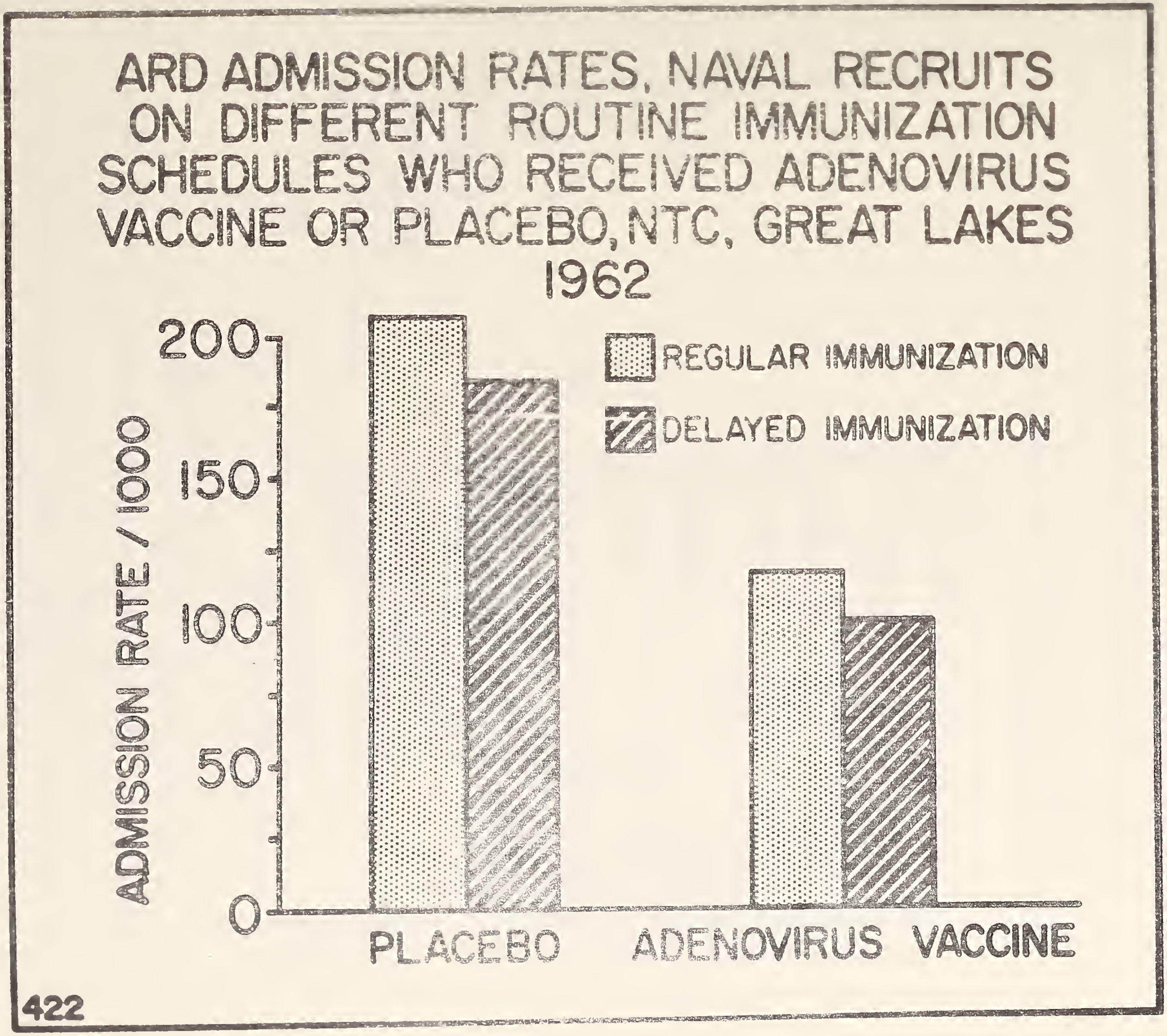

DISPENSARY VISITS NOT RESULTING IN ADMISSIONS FOR FEBRILE RESPIRATORY COMPLAINTS
RATE / 1000
24
16
8
0
REGULAR
DELAYED
OVER ALL RELATIVE REDUCTION 14.6 %
1
2
3
4
5
6
7
8
9
10
WEEK OF TRAINING
421
421

FIGURE 8

PERCENT RANDOMLY SELECTED RECRUITS ON DIFFERENT IMMUNIZATION SCHEDULES WITH ARD SYMPTOMS, NTC, GREAT LAKES, ILL. 1961





ARD ADMISSION RATES, NAVAL RECRUITS ON DIFFERENT ROUTINE IMMUNIZATION SCHEDULES WHO RECEIVED ADENOVIRUS VACCINE OR PLACEBO, NTC, GREAT LAKES 1962

FIGURE [illegible]

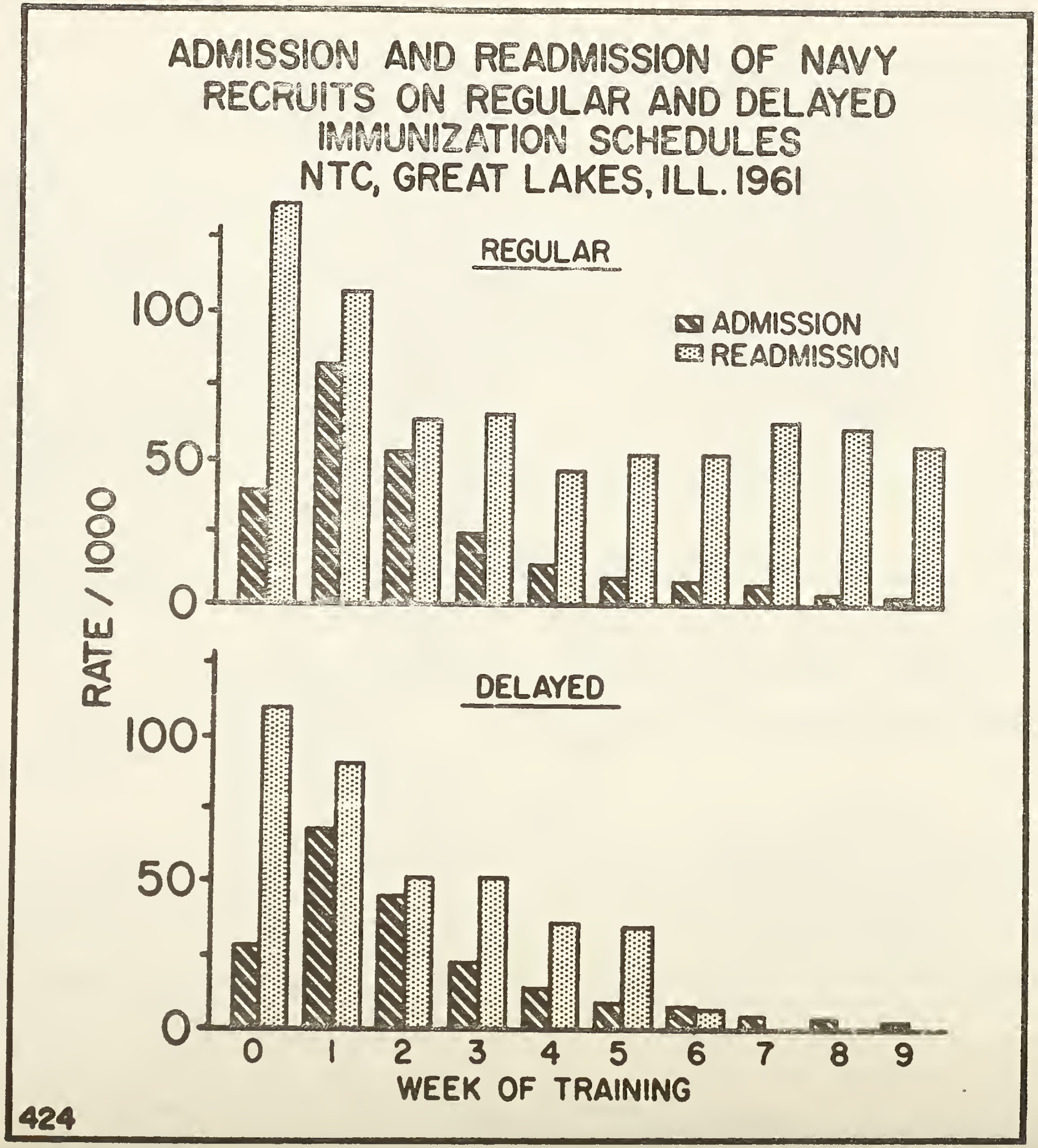

ADMISSION AND READMISSION OF NAVY RECRUITS ON REGULAR AND DELAYED IMMUNIZATION SCHEDULES
NTC, GREAT LAKES, ILL. 1961
REGULAR
ADMISSION
READMISSION
DELAYED
RATE / 1000
100
50
0
100
50
0
0
1
2
3
4
5
6
7
8
9
WEEK OF TRAINING
424

FIGURE 11

TABLE 1
Immunization Schedules Employed in Naval Recruits
Great Lakes, Illinois 1961-1962

Schedule	Initial processing period	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk	9th wk
Regular Schedule	Polio*	TAB†	TAB	TAB		Tuberculin test				
	Influenza	Smallpox	Benzathine penicillin			Tetanus & diphtheria				
	Adenovirus**	Tetanus and diphtheria				Polio				
Delayed Schedule	Polio		Benzathine penicillin			TAB	TAB	TAB		Tetanus and diphtheria
						Polio				
	Influenza					Tuberculin test				
	Adenovirus**					Tetanus & diphtheria				
						Smallpox				

*Salk vaccine

†Typhoid-paratyphoid A and B vaccine

** When given

TABLE 2
Pneumonia in Naval Recruits, on Different Immunization Schedules, NTC, Great Lakes, Ill., April-May 1962

Schedule	Population	Admissions prior survey		X-Ray survey		Admissions subsequent to survey		Total	
		No.	Rate/ 1000	No.	Rate/ 1000	No.	Rate/ 1000	No.	Ra 10
Regular	1,715	145	84.5	56	32.7	22	12.8	223	1
Delayed	1,005	34	33.8	25	24.9	8	8.0	67	
% Relative reduction		60*		24†		37.5†		48.7*	
Total	2,720	179	65.8	81	29.8	30	11.0	290	1

* P = <0.001

† P = 0.25

TABLE 3
Specific ARD Admission Rates for Adenovirus and Nonadenovirus-Associated Illness, Navy Recruits on Different Immunization Schedules, NTC, Great Lakes, Ill., Aug-Dec., 1961

Immunization schedule	ARD Admissions rate/1000 period	% Adenovirus positive CF	Specific adenovirus rate/1000	Nonadenvirus rate/1000
Regular	180.4	184/253 (72.7)	131.2	49.2
Delayed	146.3	104/147 (70.7)	103.5	42.9

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Immunologic Studies of Schistosomiasis (U)

ANNUAL PROGRESS REPORT

by

Edgar E. Baker, Ph.D

September 1968

(for the period 15 January 1967 to 30 June 1968)

U.S. Army Medical Research and Development Command

Washington, D. C. 20315

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Boston University School of Medicine

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Summary

During experiments on fractionation of extracts of adult *Schistosoma mansoni*, a precipitate formed in the extracts when they were concentrated by ultrafiltration, dialyzed against water or stored in the frozen state. Extracts of worms prepared by sonication were particularly unsatisfactory in this respect. Whenever precipitation occurred, there was loss of serologic activity and protein. Studies to determine the best methods of extraction, concentration and storage of antigens were conducted. Buffers of various molarity, pH and composition were used to extract worms. Sonication was avoided. As much material was extracted from worms in 0.01 M buffers as when 0.1 M buffers were used. Less protein and less serologically active material were recovered in extracts prepared at pH 6.0. There was no difference in serologic activity of extracts at pH 7.2 to 9.0; however, EDTA buffer pH 7.2 extracted less protein than Tes or phosphate buffer at pH 7.2. Phosphate buffer extracts at either pH 7.2 or 6.0, or an EDTA buffer pH 7.2, precipitated upon freezing. Extracts in Tes buffer at pH 7.2 could be stored in the frozen state or at 4 C with no loss of serologic activity and no visible precipitation for at least one month. Extracts made in glycine buffer at pH 9.0 were less stable when stored at 4 C than those made in Tes buffer at pH 7.2. Concentration of extracts was accomplished by lyophilization and reconstitution to 1/5 the original volume without loss. Extracts could be dialyzed against very dilute buffers for 1 day without loss but not against water.

Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care". as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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Last year fractionation of sonicated extracts of adult schistosomes by filtration through Sephadex G-200 was reported. The fractions obtained each contained fewer antigens than were present in the original crude extracts, but none of the fractions contained only a single antigen. Most antigens were found either in the material excluded from G-200 or in the first fraction to be eluted from the column. This year, as in the past, Dr. Elvio Sadun, Walter Reed Army Institute of Research, Washington, D. C., supplied lyophilized adult *Schistosoma mansoni*.

Attempts to separate crude extracts on the basis of stability to freezing and thawing: In the course of previously reported experiments, it was observed that a precipitate occurred in most extracts after freezing and thawing, concentration by ultrafiltration (Diaflo UM-1 or XM-50 membranes) or dialysis against water. Although the washed precipitate did not migrate in agar gel plates, it was found to be antigenic when injected into rabbits and the antibody response was indistinguishable from that obtained by injection of the whole crude extract. Repeated sonication of the precipitate using a Branson sonifier with a 6 amp. input yielded material which remained in solution after centrifugation at 22,000 X G for two hours. This material formed three zones of precipitate in agar gel when tested against rabbit antiserum (prepared as described in a previous report).

The observations above suggested that some separation of antigens might be effected by dividing crude extracts into a fraction which did not precipitate upon freezing and subsequent thawing and a fraction that did precipitate upon freezing and thawing but which could be solubilized by sonication. In order to test this hypothesis, 770 mg of adult worms were suspended in water, ground in a Ten Broeck tissue homogenizer, and allowed to stand in the cold for one hour. The extract then was separated by centrifugation. Four such extracts, each of 25 ml, were made and combined. The combined extracts contained 4 antigens as determined by agar gel and immunoelectrophoresis using rabbit antiserum to whole worms. Two of these antigens also reacted with infected-monkey serum. The water extract was dialyzed and lyophilized. A total of 88 mg was obtained. The extracted worms then were suspended in 0.005 M phosphate buffer, pH 7.2, and sonicated for one minute (input 6 amp). This treatment was repeated four times. The residue was then frozen and not studied further.

The supernatants collected by centrifugation were frozen overnight. The next day the precipitate which formed after thawing was collected by centrifugation, dissolved by sonication and refrozen. Each day the combined precipitates obtained after freezing were redissolved by sonication. The process was repeated 12 times. The sonically solubilized material totalled 18.4 mg after lyophilization and contained 4 antigens identical with the antigens of the unsonicated extract. From these data it is evident that the bulk of the antigens is to be found in the soluble fraction with or without sonication. Unfortunately, each time the soluble fraction was stored in the frozen state, some precipitate formed. This precipitate could be redissolved by sonication. After the sonication process was repeated the twelfth time, the precipitate which formed after freezing was redissolved by sonication and lyophilized. It totalled only 3 mg and contained 3 antigens which were identical with 3 of those also found in the soluble fraction. Thus, it was not possible under these conditions to separate the crude extract into a fraction stable to freezing and thawing and an antigenically different fraction precipitable by freezing and thawing.

Fractionation with Sepharose 4B: Since in previous experiments, most antigens were recovered from Sephadex G-200 in either the excluded fraction or the first eluted fraction, it was thought that better separation might be effected by the use of Sepharose 4B (fractionation range MW 300,000 to 2,000,000). 67 mg of water soluble material obtained in the previous experiment (containing 14.4 mg N) was put on a Sepharose 4B column measuring 1 x 60 cm. 0.005 M phosphate buffer was run through the column at the rate of 4.2 ml per hour. 1.6 ml samples were collected and their absorption at 280 mμ determined. A plot of the data revealed three peaks. However, serological testing of each sample from the column revealed no separation of antigens. The fraction containing the greatest number of antigens (3) was eluted immediately after the serologically inactive excluded material.

Preparation of extracts without sonication and with fractionation on Sepharose 4B: In the above experiments most of the antigenic material was recovered in the water extract. It was also found that material solubilized by sonication precipitated upon freezing. Thus, it was thought that decreased losses of antigens might be obtained if sonication were avoided entirely.

The following experiment was conducted to test this possibility. 505 mg of defatted adult worms were extracted by grinding for two minutes in cold 0.02 M EDTA buffer pH 7.2 in a Ten Broeck tissue homogenizer. The material was allowed to extract in the cold for two hours and the extract separated by centrifugation. The extraction was repeated until no more trichloracetic acid precipitable material was extracted (a total of 5 extractions). The extracts were pooled, concentrated on a Diaflo UM-1 ultrafilter (considerable material became insoluble upon filtration), dialyzed against water and lyophilized. 148 mg of material were recovered. 5 antigens which reacted with hyperimmune rabbit serum of which 3 reacted with infected-monkey serum were found. 91 mg of the EDTA extract were fractionated on a Sepharose 4B column (1 x 60 cm) by elution with 0.02 M EDTA buffer pH 7.2. Two peaks as determined by adsorption at 280 mμ were obtained in material eluted from the column. However, these peaks did not correlate with antigenic composition. After serologically testing individual 1.6 ml samples, pools were prepared as follows: Fraction 1 was the excluded material in the first sharp peak as measured at 280 mμ. It contained 2 antigens which reacted with hyperimmune rabbit serum of which 1 reacted with infected-monkey serum. Fraction 2 was the second broad peak. It contained 3 antigens which reacted with hyperimmune rabbit serum of which 1 reacted with infected-monkey serum. Fraction 3, containing the material eluted last, had only a single weakly reacting antigen. These fractions were titrated and compared serologically. These data are presented in Table I.

Fraction 1 and 2 were each rechromatographed on Sepharose 4B in an effort to separate the antigens contained in each. Recycling of Fraction 1 did not effect separation of the 2 antigens in it and most of the material was lost. Rechromatography of Fraction 2 likewise did not effect separation of antigens although there was some increase in specific activity. (See Table I).

As in the preceding experiments, sonication of the worm residue in 0.02 M EDTA pH 7.2 buffer at 6 amps for 2 minutes using a Branson sonifier yielded material containing 4 antigens identical with 4 of those found in the water extract. In an attempt to fractionate this material none of the material could be eluted from the Sepharose 4B column.

On the basis of the above experiments it was thought that with larger amounts of starting material, it might be possible to collect smaller samples and perhaps obtain better separation. A large batch of adult worms was extracted in 0.02 M EDTA buffer pH 7.2 as outlined above. The EDTA extract was put on a Sepharose 4B column (2.5 cm x 60 cm) with rather disappointing results. Only about 40% of the total material put on the column was recovered. Although elution resulted in several peaks based on absorption at 280 mμ, the antigens appeared to be scattered in the various fractions. One fraction seemed to contain considerable amounts of the major antigen reacting with infected-monkey serum. However, upon titration in Crowle plates (agar gel) there was not much improvement in activity over the original EDTA extract.

Effect of pH upon material precipitated by freezing: In confirmation of Kent (Ann. New York Acad. Sci. 113: 100, 1963), any handling of fractions resulted in loss due to formation of a precipitate. It has been observed that if material is preserved by freezing, a considerable amount of insoluble material separated upon thawing. These residues were all kept. They were combined and re-extracted with 0.15 M phosphate buffer pH 7.8 because it appeared that pH 7.0 to 7.2, which was usually used for extraction, was borderline in terms of solubility of antigens. A relatively large amount

Table I

Fractionation with Sepharose 4B

Antigen Fraction	Recovered from	Total Yield	Agar Gel Zone Number*				
			1	2	3	4	5
EDTA Extract	505 mg defatted worms	148 mg	625**	78	1250	313	2500
1	90 mg EDTA extract on Sepharose	34 mg	Not Titrated				
2		26 mg	125	8		125	
3		19 mg	Not Titrated				
1a	30 mg Fract. 1 on Sepharose	0.7 mg	250				
2a	23 mg Fract. 2 on Sepharose	6.5 mg	63	8		31	

* Zone number designation made by numbering zones in reaction of the EDTA extract with hyperimmune rabbit serum. Numbers start from zone located closest to antiserum reservoir and progress outward toward antigen reservoir. Zones 1, 2, 4 and 5 also react with infected-monkey serum.

** Numbers designate lowest concentration in µg per ml of antigen solution which would form visible precipitate zones when tested with hyperimmune rabbit serum in agar gel tests when 10 µl of solution were used in each reservoir.

of material was obtained with high serologic activity. It could be diluted to 15 µg per ml and still give a zone of precipitate in Crowle plates. After storage in the frozen state this material tended to precipitate but could always be brought into solution by the addition of a glycine buffer, pH 9.0 (to 0.01 M). In order to determine whether high pH values inactivated antigens, material was adjusted to pH 9.0, 9.5, 10.0 and then titrated in Crowle plates. It was found that at pH 9.5 and 10.0 activity was as great or greater than at lower pHs. However, as will be discussed below there is some loss of activity upon storage of extracts at pH 9.0 for prolonged periods.

Ultracentrifugation in a Sucrose Gradient: Although the material extracted at a high pH from the combined residue is not entirely representative of an extract of whole adults it contained at least as many components as did the original EDTA extract. It was decided to use it for further experiments after dialysis to remove salts. In view of the poor separation on Sepharose, separation by ultracentrifugation in a sucrose gradient was investigated. Since the antigens appeared to be soluble at pH 9.0, a preliminary experiment was conducted at pH 9.0 using a gradient of 20% to 5% sucrose. When the material was centrifuged in 30 ml tubes at 90,137 X G (25,000 RPM) in a Spinco Model L with a SW 25 rotor for 18 hours, it entered the gradient far enough to suggest molecular weights for the various antigens in the neighborhood of 100,000. Unfortunately, all antigens seemed to fall in the same range of molecular weight and no separation was effected.

A Study of Methods of Preparing Extracts: All of the experiments outlined above as well as the experience of other investigators showed that considerable losses occurred in handling extracts. In view of these difficulties it was felt that before proceeding further it would be necessary to re-examine the methods of extraction, concentration and storage of extracts of schistosomes.

Systematic studies were undertaken to determine the effect of pH, buffer composition and concentration upon extraction and subsequent handling of extracts. In all experiments 10 mg of defatted worms were suspended in 2 ml of buffer and stirred for two hours at 4 C. The suspensions were then centrifuged at 22,000 X G for one hour. Usually three extracts were made but held separately with most testing done on first extracts only.

To test efficiency of extraction, several tests were conducted using buffers of various pH values and composition. Duplicate tests were done with buffers of 0.1 M and 0.01 M. The protein content of extracts was determined by measurement of the difference in optical density at 233 and 224 mµ to avoid interference by nucleic acid (Groves, Davis and Sells, Anal. Biochem. 22: 195, 1968). Serologic activity was determined by titration (serial 2 fold dilutions) in agar gel plates by the Crowle method. Results are tabulated in Table II for phosphate buffer at pH 6.0 and 7.2, EDTA buffer at pH 7.2, Tes (N-Tris (hydroxymethyl) Methyl-2-Amino Ethane

Sulfonic Acid) buffer at pH 7.2 and 8.0 and Glycine buffer at pH 9.0.

In the first extracts, between 0.8 mg and 0.93 mg protein per ml of extract was obtained at pH values between 7.2 and 9.0. Considerably less protein was obtained at pH 6.0 and a little more at pH 9.0. EDTA buffer extracted somewhat less protein than either phosphate or Tes at pH 7.2. This difference has been observed on several occasions. Serologically, the extracts made at pH 7.0, 7.2 and 8.0 were all similar with a maximum of five antigens detectable in the undiluted first extract. The extracts could be diluted to more than 1:64 before all serologic activity disappeared. In first extracts at pH 6.0 the maximal number of antigens that could be detected was four, one less than at the other pH values tested. Activity in the pH 6.0 extract was detectable only to a dilution of 1:16. Table II shows data for extracts using 0.01 M buffers. Duplicate experiments using 0.1 M buffers yielded the same results.

In another experiment 20 mg of worms per ml of buffer with a total of five extractions were used. In contrast, the total yield of protein was about one third less than that obtained when 5 mg of worms were extracted per ml of buffer.

Stability of Extracts: 1. Freezing: As reported in previous experiments, freezing of extracts led to the formation of a precipitate. When this occurred, there was considerable loss of serologic activity as well as of protein. In an effort to determine the cause of precipitation, 2 portions of each of the first extracts named in Table II were frozen immediately after preparation. One portion was thawed and refrozen daily for one month. The other portion was held in the frozen state for one month and then thawed.

In the repeatedly frozen and thawed samples there was visible precipitate only in the tubes containing phosphate buffer and it was detected only after thawing the fifteenth time. After repeated freezing and thawing some serologic activity was lost (one antigen). However, there was no loss in serologic activity of the major antigen. This seems to indicate that the antigen responsible for titration of serologic activity out to the highest dilution is resistant to repeated freezing and thawing. It is not possible to determine whether small losses occurred in other antigens.

Identical results, with one exception, to those cited above were found in the samples held frozen for one month and then thawed. There was no loss in serologic activity in either of the extracts made in Tes buffer.

In other experiments, daily freezing and thawing of either 0.05 M or 0.1 M EDTA extracts resulted in precipitation in three days. When 0.1 M phosphate buffer pH 7.2 was used, precipitation occurred after freezing

Table II

Effect of Buffer Composition on Extraction of Antigens

		Protein Protein (mg/ml)				Serology			
						First Extract		Second Extract	
0.01 M Buffer	pH	First Extract	Second Extract	Total (mg)	% Yield	Maximum No. Antigens Detected	Activity Detected to Dilution	Maximum No. Antigens Detected	Activity Detected to Dilution
Phosphate	6.0	0.56	0.06	1.24	12.3	4	1:16	1	1:4
Phosphate	7.2	0.93	0.26	2.38	22.2	5	1:64	2	1:16
EDTA	7.2	0.80	0.07	1.74	15.9	5	1:64	2	1:16
Tes	7.2	0.85	0.20	2.10	21.4	5	1:64	2	1:16
Tes	8.0	0.93	0.20	2.26	21.5	5	1:64	2	1:8
Glycine	9.0	1.11	0.22	2.66	25.7	5	1:64	2	1:8

and thawing five times. In contrast, no precipitation occurred in 0.1 M Tes pH 7.2 or 0.1 M glycine pH 9.0 even after daily freezing and thawing for two weeks.

2. Sonication: All extracts prepared by sonication of worms or worm residues yielded large amounts of precipitate after being frozen overnight. This occurred regardless of the type or pH of buffer used.

3. Dialysis: In past experiments, it was customary to dialyze all antigen preparations against water in order to remove buffer salts prior to lyophilization. It was noted that a precipitate always occurred. Since it was thought that a pH change of the extract during dialysis against water might be responsible for the formation of the insoluble substance, experiments were performed using extracts prepared in 0.01 M Tes buffer pH 7.2 and 0.01 M Glycine buffer pH 9.0. Portions of each extract were dialyzed overnight against 0.005 M, 0.0025 M, and 0.00125 M buffer. There was no precipitate in any extracts dialyzed against 0.005 M or 0.0025 M buffer and there was no loss in serologic activity of these extracts as compared to each extract before dialysis. However, continued dialysis of the extracts another day resulted in precipitation and loss of serologic activity as well. Extracts dialyzed overnight against 0.00125 M buffer did precipitate.

4. Ultrafiltration: Initially, ultrafiltration appeared to be a quick and efficient means of concentrating solutions containing proteins. The Diaflo ultrafilter (Amicon Corp.) fitted with an UM-1 membrane was used to concentrate extracts. A yellowish precipitate occurred on the surface of the membrane. Use of an XM-50 membrane reported by the Amicon Corp. to be of a different physical make-up and greater porosity than the UM-1 membrane, was no better. Concentration by ultrafiltration consistently led to precipitation of antigens. This precipitate could be redissolved by sonication but with loss in activity.

5. Lyophilization: Lyophilization was examined as a means of concentrating and storing extracts. Two portions of each of the first extracts listed in Table II were lyophilized. One portion was reconstituted after one day and the other portion was reconstituted after storage in the cold for one month. Upon reconstitution to a concentration comparable to the original extract, no precipitate (or only a slight precipitate in a few experiments) was observed. After lyophilization and reconstitution one day later, there was no loss of serologic activity. After one month storage, there was a slight loss of serologic activity but of doubtful significance. The loss was most notable in extracts prepared at pH 8.0 and pH 9.0. (See Table III). It was found possible to reconstitute samples to about 1/5 the original volume without loss. Thus, lyophilization appeared to provide the only effective method of concentration so far found.

6. Temperature: Extracts were prepared in 0.01 M Tes pH 7.2 buffer and in 0.01 M Glycine pH 9.0 buffer. Each of these was sterilized by Millipore filtration and portions stored at 4 C, 20 C and 37 C for 30 days. All samples remained sterile throughout the test period. Aliquots were tested after 4, 10, 14 and 30 days' storage by titration in agar gel plates (Crowle method). Results of these tests may be seen in Table III. There was no antigenic loss in 30 days in extracts made at pH 7.2 and stored at 4 C. Material stored at 20 C showed a considerable drop in serologic activity after 10 days. Storage at 37 C produced some loss of activity after 4 days and with a greater loss after 10 days. Extracts prepared at pH 9.0 were less stable. There was some loss of serologic activity within 4 days at 4 C and at 20 C and even more in 14 days. Storage at 37 C resulted in nearly complete loss of activity in 4 days. Storage at 4 C in Tes buffer at pH 7.2 was more effective in preservation of antigens than storage at the higher pH or at higher temperatures. It is notable, however, that the extracts contain some remarkably stable antigens which withstand storage for long periods at relatively high temperatures.

Fractionation on DEAE Cellulose: Preliminary experiments showed that if large amounts of DEAE are used the antigens cannot be eluted even by high salt concentrations. Ratios of DEAE of 7.5 mg to 1 mg of protein or less must be used. These experiments are being continued.

Discussion

Several experiments were conducted in which adult schistosomes were extracted with and without sonication. Both phosphate and EDTA buffers were used. Upon storage in the frozen state or concentration by ultrafiltration, precipitates formed with loss of serologic activity even though in most cases the precipitates could be redissolved with the aid of sonication. Attempts to fractionate the extracts on Sepharose 4B were unsatisfactory and large losses occurred. It may be that the conditions which led to precipitation during storage or concentration also resulted in loss of solubility in the column and the resulting losses of material. The difficulties encountered led to the conclusion that although the conditions for extraction employed were widely used by other investigators, they may have been responsible for the lack of stability of antigens observed. Thus, it was considered necessary to reinvestigate methods of extraction, concentration and storage of worm extracts.

Systematic studies of extraction procedures and methods of handling extracts have shown that stable extracts of high serologic activity can be prepared by careful control of experimental conditions. It appeared that extracts

Table III

Stability of Antigens During Storage at Different Temperatures

Preparation	Storage Temperature	Time of Storage (days)				
		0	4	10	14	30
pH 7.2 Tes	Frozen	-	-	-	-	1:64*
	Lyophilized	-	-	-	-	1:64
	4 C	1:64	1:64	1:64	1:64	1:64
	20 C	1:64	1:64	1:64	1:16	1:8
	37 C	1:64	1:32	1:32	1:4	-
pH 9.0 Glycine	Frozen	-	-	-	-	1:64
	Lyophilized	-	-	-	-	1:32
	4 C	1:64	1:32	1:32	1:16	1:16
	20 C	1:64	1:32	-	1:16	1:16
	37 C	1:64	1:16	-	1:8	-

* Highest dilution at which a single zone of precipitate formed.

of adult worms should be made without sonication. 0.01 M buffers extracted as much antigenic material as buffers of stronger salt concentration. Although dialysis against water caused insolubility of antigens, extracts could be dialyzed against dilute buffers for short periods of time.

Antigenic extracts in Tes buffer, pH 7.2, remained in solution after repeated freezing and thawing. In fact, these extracts could be stored at -20 C with no loss of serologic activity for at least a month. The major antigen in Tes extracts could be detected in dilution as high as 1:64 and it remained just as active in these extracts stored at 4 C for one month or at 20 C for 10 days. However, there was considerable loss of activity in extracts stored in Glycine buffer pH 9.0. Material could be effectively concentrated by lyophilization and antigens remained active when stored in the lyophilized state at 4 C at least a month.

Conclusions and Recommendations

1. Extracts of adult worms can be prepared with 0.01 M Tes buffer pH 7.2, which are stable for at least a month in the frozen state or at 4 C.

2. Tes extracts can be concentrated by lyophilization without loss.

3. Tes extracts can be dialyzed against dilute buffer for up to one day without loss but not for longer periods of time.

4. Preliminary results of ultracentrifugation in sucrose gradients suggest a range of molecular weights of antigens in the neighborhood of 100,000. These data indicate that Sephadex G 100 might be most suitable for fractionation. The fact that all antigens appear to move into the gradient as a single zone suggests some interaction may occur. It is suggested that higher pH values and salt concentrations be used for dilution.

5. Preliminary experiments with DEAE indicate that fractionation of Tes buffer extracts may be possible. However, the difficulty in elution suggests that other ion exchangers should be studied.

Acknowledgment

The work presented in this report was conducted with the assistance of Dr. Roberta Whiteside and Miss Margaret Johns. We wish to thank Dr. Elvio Sadun, Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D. C., for supplying lyophilized S. mansoni, for conducting serological tests, and for his very helpful advice.

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13 ABSTRACT

During experiments on fractionation of extracts of adult Schistosoma mansoni, a precipitate formed in the extracts when they were concentrated by ultrafiltration, dialyzed against water or stored in the frozen state. Extracts of worms prepared by sonication were particularly unsatisfactory in this respect. Whenever precipitation occurred, there was loss of serologic activity and protein. Studies to determine the best methods of extraction, concentration and storage of antigens were conducted. Buffers of various molarity, pH and composition were used to extract worms. Sonication was avoided. As much material was extracted from worms in 0.01 M buffers as when 0.1 M buffers were used. Less protein and less serologically active material were recovered in extracts prepared at pH 6.0. There was no difference in serologic activity of extracts at pH 7.2 to 9.0: however, EDTA buffer pH 7.2 extracted less protein than Tes or phosphate buffer at pH 7.2. Phosphate buffer extracts at either pH 7.2 or 6.0, or an EDTA buffer pH 7.2, precipitated upon freezing. Extracts in Tes buffer at pH 7.2 could be stored in the frozen state or at 4 C with no loss of serologic activity and no visible precipitation for at least one month. Extracts made in glycine buffer at pH 9.0 were less stable when stored at 4 C than those made in Tes buffer at pH 7.2. Concentration of extracts was accomplished by lyophilization and reconstitution to 1/5 the original volume without loss. Extracts could be dialyzed against very dilute buffers for 1 day without loss but not against water.

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FACTORS INFLUENCING THE PATTERN OF THE IMMUNE RESPONSE (U)

ANNUAL PROGRESS REPORT

by

Geoffrey Edsall, M.D.

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(For the period 1 March 1967 to 30 June 1968)

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SUMMARY

The purpose of these studies is to define the pattern of the immune response in order to improve the efficacy of immunization procedures and to prepare and characterize more highly purified antigens and combinations of antigens, so to facilitate dosage reduction and minimize reactions. (U) This year, serum samples from the 56-day and 375-day bleedings of volunteers inoculated in 1966 with 4 different tetanus toxoids were titrated for antitoxin. One-half got 2nd injection of toxoid on Day 28, the other half on Day 56. After one injection, Day 56 titers were 50-100% higher than at Day 28 except in the maximal-response group. 56-day levels after 2 injections of toxoid were 12-36 times higher than after 1 injection. Differences in level appeared more closely correlated with [Antigen] than with [$AlPO_4$]. 62 of 63 individuals tested at 1 year (after 2 injections) still carried protective titers. The level was related more to the 1st injection than to the 2nd. A group was started on one injection of the best of the 4 toxoids, to determine the long term protection afforded during 1 year. Studies on passive tetanus hemagglutination titers found in the absence of toxin neutralizing antibody showed they were non-specific, and apparently were due to an unidentified serum factor. Calculations from 182 tetanus antitoxin levels in well immunized children indicated that after 4 doses of toxoid only 1 child in 1000 would have a level below the threshold of protection in the following 10 years. The slow and fast components of tetanus toxin were further characterized. A hypothesis which may explain some of the discrepancies in active mouse protection tests of typhoid vaccines is now being tested.

FOREWORD

This report summarizes work performed under Contract DA-49-193-MD-2144 with the Office of The Surgeon General, United States Army, and sponsored by the Commission on Immunization, Armed Forces Epidemiological Board. The contract with Harvard University is administered through the Harvard School of Public Health. The work is actually carried out at the State Laboratory Institute of the Massachusetts Department of Public Health in Forest Hills, a few miles from the Harvard Medical-Public Health area. William C. Latham has been primarily responsible for much of the work, along with Robert G. Chisholm, and Miss L. H. Wetterlow has done the typhoid vaccine studies. Space does not permit specific acknowledgment to each of the many members of the Institute or Contract staffs who have contributed. In addition, invaluable -- and gratifying -- collaboration with colleagues outside the Institute has included:

Dr. Kenneth W. Newell, WHO Geneva
Prof. Paul Beaver, Dr. Robert MacLennan,
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Dr. Hans Cohen and Dr. J. D. van Ramshorst,
   Utrecht, the Netherlands
Dr. Thomas C. Peebles, Weston, Massachusetts

for whose help or advice, or both, the undersigned and their associates are deeply grateful.

In conducting the research described in this report, the investigators have adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research".

The general problem to which this study is directed is the simplification and improvement of immunization. To this end the following specific major objectives are being pursued:

(a) To characterize the pattern of the response to immunizing agents and the factors regulating this response, as regards to dose, timing of injections, use and choice of adjuvants, physical state of antigen, etc., so as to provide a sound basis for simpler or more effective immunization procedures for personnel under military jurisdiction. Special attention will continue to be given to (i) the initial phases of the primary immune response and (ii) the maintenance of long-term immunity.

(b) To develop methods for purification of immunizing agents, and to carry out related studies, as a basis for reducing the incidence or severity of reactions to immunization.

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Factors Influencing the Pattern of the Immune Response

Geoffrey Edsall, M.D., Principal Investigator

Work supported by Contract MD-2144 during 1967-68 falls into the following areas:

1. Factors influencing the early primary immune response

a. Studies in animals: During the period in question animal studies were suspended because of the uncertainty that the findings to date could be transferred to man, without extensive preliminary safety trials. Since human subjects are limited in number, plans were therefore oriented, for the present, to trials of conventional antigen preparations.

Animal studies will, however, be resumed in the fall of 1968, with pilot experiments on Johnson's poly A-U adjuvant (1).

b. Studies in man: Last year we reported on the early primary response of 150 humans to inoculation of 4 tetanus toxoid preparations differing only in the concentration of antigen and of the mineral adjuvant $AlPO_4$ (See Table 1). Subjects with a measurable titer at Day 0 or Day 7 were excluded. The 14-, 21- and 28-day responses showed a steadily increasing spread between the "low-low" preparation (A) and the "high-high" preparation (D). Of the two intermediate preparations, 'B' (high-antigen-low adjuvant) appeared to be somewhat better.

Re-examination of the data, in conference with the Tulane collaborative group, brought to light a trend to a biphasic distribution of responses at 28 days, confirmed even more strikingly in the 1967 study group (see below). The possibility that this could be related to menstrual cycle (since all subjects were female) was attractive but later proved doubtful when similar findings were noted, in a Ft. Detrick study done some years ago -- in males. Acceptable hypotheses to account for this non-normal distribution, and an experimental test of any such hypothesis, are now being screened in collaboration with the Tulane-Valle ICMRT group.

Sera drawn at ±56 days and at about 1 year (actually 373 to 378 days) after the initial injection were titrated. At 56 days one-half of the group had gone 28 days since their 2nd toxoid injection (which was part of the compact with the subjects -- i.e., that they would be immunized to tetanus). The other half received their 2nd injection immediately after the 56-day bleeding. Each subject received the same toxoid in the 2nd dose that she had had in the first. The 56-day

Table 1

Cali Study I
Characteristics of Toxoids Used

Toxoid Lot	Amount per dose		
	Toxoid (Lf)	$AlPO_4$ (mg)	al
AL6A	5	0.77	0.28
AL6B	25	0.77	
AL6C	5	3.86	0.85
AL6D	25	3.86	

Table 2

Geometric Mean Tetanus Antitoxin Titers in Subjects receiving 2 injections Tetanus Toxoid Lots AL6A, B,C or D

	Bled (Days after 1st Injection)					
Lot	14	21	28	56[1]	56[2]	375[3]
A	.0018	.0026	.0038	.0066	.17	.056
B	.0027	.0046	.0085	.015	.56	.29
C	.0021	.0043	.0080	.019	.23	.12
D	.0032	.014	.029	.032	.69	.40

[1] Before 2nd injection
[2] 28 days after 2nd injection
[3] 10-11 months after 2nd injection

titers (see Table 2) showed that

(a) in subjects not yet given a 2nd injection, the geometric mean levels were 50-100% higher -- in 3 of the 4 groups -- than the means at 28 days, suggesting that the "primary" response to an adsorbed type tetanus toxoid peaks some time after one month. (The term "primary" is of course empirical in this situation, since there is abundant evidence from other studies that at this stage in time, the response is a complex of primary and secondary responses). Only in Group D was there little increase from 28 to 56 days -- suggesting the possibility that with this potent stimulus the peak response came earlier. Jerne and his colleagues have observed this in plaquing studies, but the analogy is not necessarily relevant, since we have as yet no evidence that IgM antibody plays any significant role in tetanus toxin neutralization.

(b) In the subjects responding to a 2nd dose of toxoid, the 56-day G.M. levels were 12 to 36 times higher than in the matching group 56 days after a single injection. Lots B and D (high antigen content) yielded the highest G.M. levels, suggesting that when sufficient carrier was present to insure 'capture' of the antigen, then more carrier added little, whereas more antigen added significantly, presumably increasing the number of cells committed to antibody formation. However, with only 12 to 18 subjects per group (8 groups) such inferences remain speculative.

The one-year titers showed that of the 63 individuals tested who had received 2 toxoid injections, only one (in Group D!) had a titer below the usually accepted threshold of protection. The titers showed no major change in the relative G.M. levels (A:B:C:D = 1:5:2:7) as compared to the 28 day ratios (1:2:2:8) except for Group B. The absence of any general increase in the spread between A and D suggests that the dose-response slope with the second dose was relatively flat, as Ipsen's Commission-sponsored studies of 15 years ago found. Stated differently, this assumption is consistent with the working hypothesis that the height (and perhaps the duration) of the total immune response is largely determined by the "immunizing efficacy" of the initial injection. This hypothesis has limits, since Sterzl (2), Siskind et al (3), Cerny et al (4) and perhaps others, have shown that 'excess' antigen can depress the late primary or inhibit the early secondary response. Therefore, two further parameters of the primary and secondary response to a defined protein antigen in man require clarification: The role of antigen dose in the second stimulus, and the possible limiting effect of excess antigen in the first stimulus. Protocols for the former study are being organized; the latter study may present difficulties, since "excess" antigen -- in the case of tetanus toxoid -- may well prove to be more than is acceptable in normal human subjects.

Because of the excellent primary response to a single dose of the "high-high" toxoid D, it was postulated that a preparation such as this might be effective as a single-dose immunizing injection in persons unlikely to be seen again for a second injection. If indeed it served this purpose, then it could be an all-purpose preparation since it would "prime" the recipient to respond effectively to a corresponding stimulus for years and possibly for life. Therefore, again in collaboration with the Tulane-Valle ICMRT staff, a group of 40 unimmunized subjects was given a single dose of Toxoid D, bled on Days 0, 7 and 28, and kept under observation without further tetanus toxoid injections. (In the event that a subject incurs a tetanus-prone injury she will be treated appropriately and removed from the group). At the end of one year, each of these subjects will be re-bled and given a second dose of toxoid to complete her immunization.

The 28-day titers in this group (after excluding all subjects with Day 0 or Day 7 titers suggesting prior experience with the antigen) are shown in Table 3. Here again a biphasic distribution appears; the average G.M. titer is 0.08 unit, about twice that of the previous comparable group.

c. Techniques for measurement of antibodies and antigenic potency

(1) Study of the nature of false positive tetanus antitoxin titers obtained in passive hemagglutination tests -- In 5 recent studies on human populations (2 North American and 3 Colombian), 114 subjects out of 360 with no detectable neutralizing tetanus antitoxin had low but positive titers by passive hemagglutination (HA) test (mode = 0.005 AU-equivalent per ml). Several attempts were made to account for and if possible correct this deficiency of the HA method, but this effort was handicapped by the fact that only very small serum samples (1 to 3 ml) were available from these studies. However, with the cooperation of colleagues in the field, we succeeded in obtaining a large blood donation from each of 3 individuals who had relatively high HA titers but no neutralizing antibody. Plasma from single donations of blood was made available from 2 subjects of the Viejes Study, A. G. and J. T., and from one subject of a study being carried out by Drs. Ganem and Madoff at the Lemuel Shattuck Hospital, Y.M. Samples were confirmed as having no detectable neutralizing power for tetanus toxin in mice (< 0.0025 AU/ml), whereas by HA test, A.G. had 0.06, J. T. 0.03 and Y. M. 0.12 HU/ml. J.T. gave uncertain endpoints and was not used.

Specificity test: Specific antigen (tetanus toxoid) was added to aliquots of the sera to see whether it would specifically inactivate the agglutinin. An indifferent antigen (diphtheria toxoid) was also added to other aliquots as a control. Similar additions were also made to standard equine antitoxin (STD) and to Tetanus Immune Globulin

Table 3

Tetanus Antitoxin Titers
28 days after injection of Toxoid AL6D

(October 1967)

		Number of Subjects	
Code	Titer Units	No Measurable titer, Day 7	Detectable titer, Day 7
0	< .0025	2	0
1	.0025-.005	1	0
2	.005-.01	2	0
3	.01 - .02	5	0
4	.02 - .04	2	0
5	.04 - .08	0	0
6	.08 - .16	2	0
7	.16 - .32	7	0
8	.32 - .64	3	0
9	.64 - 1.2	4	1
10	1.2 - 2.5	0	0
11	2.5 - 5	1	0
12	5 - 10		1
13	10 - 20		3
14	20 - 40		2
		29	7

(TIG 3D). HA tests against tetanus-toxoid-sensitized cells were carried out in these mixtures, with results as shown in Table 4. Titers were virtually unchanged in the false-positive-plasmas AG and YM, indicating non-specificity of the agglutinins. Results were equivocal with the equine standard, STD, but with purified TIG, specific antigen blocked most of the agglutinin, whereas indifferent antigen left it unchanged.

Effect of mercaptoethanol on the false agglutinin: Plasmas AG and YM were treated with 2 ME, which caused a 4-fold reduction in HA titer (2 tubes).

Effect of pure antigen on the false agglutinin: Tanned cells were sensitized with a crude tetanus toxoid antigen and with a highly purified one and the effect of each was tested on the 4 sera AG, YM, STD, and TIG, with results as shown in Table 5. There was no significant difference between pure and crude antigen with the false agglutinins in AG and YM, but with the true antitoxins, pure antigen "underestimated" the HA titer 8 to 16 fold.

Can the false agglutinin be demonstrated with sensitized latex particles? Tetanus-Latex agglutinations were carried out on the plasmas with the following results: JT: 0.125, AG: 0.125, YM: 0.25 HU/ml. The false tetanus agglutinin is therefore not acting on an erythrocyte factor.

Conclusions from the above are:

1. Activity of the "false" agglutinin is not abolished by treating with specific antigen.

2. Some of this agglutinin appears labile to 2 ME.

3. The false agglutinin is indifferent to the purity of the antigen but true antitoxins show lower HA titers with purer antigens.

4. The false agglutinin acts on sensitized latex particles as well as on sensitized erythrocytes.

It does not, however, limit the usefulness of the HA test as a rapid (2 to 3 hr.) method of determining whether an injured person should receive Tetanus Immune Globulin. This agent need be administered only to those persons showing less than 0.01 AU-equivalent per ml by the HA test. This is so because the false positives very rarely reach this level. An alternative method recommended for this purpose, the double-diffusion technique in agar gel of Alexander and Moncrief (5), is less suitable, we believe, because it will identify only the highly immune, since its sensitivity does not go below one unit.

Table 4

Suspect Hemagglutination Reactions

Specificity Test	Change in Endpoint Tube	HU/ml*
1. A.G. - 0.06 HU/ml		
0.9 Lf tet. toxoid added at 1:4, or 0.015 HU/ml	-1	0.03
0.3 " " " " " " " "	0	0.06
0.9 " diph. " " " " " "	-1	0.03
0.3 " " " " " " " "	0	0.06
2. Y.M. - 0.12 HU/ml		
0.9 Lf tet. toxoid added at 1:4, or 0.03 HU/ml	0	0.12
0.3 " " " " " " " "	0	0.12
0.9 " diph. " " " " " "	+1	0.24
0.3 " " " " " " " "	0	0.12
3. Standard - 5 AU/ml		
0.9 Lf tet. toxoid added at 1:320, or 0.016 AU/ml	-4	0.3
0.3 " " " " " " " "	+1	10.0
0.9 " diph. " " " " " "	0	5.0
0.3 " " " " " " " "	+2 or >	20.0 or >
4. TIG 3D - 5 AU/ml		
0.9 Lf tet. toxoid added at 1:320, or 0.016 AU/ml	-4	0.3
0.3 " " " " " " " "	-2	1.25
0.9 " diph. " " " " " "	0	5.0
0.3 " " " " " " " "	0	5.0

AG + 2ME: - 2 tubes
YM + 2ME: - 2 tubes

* In the original, undiluted serum.

Table 5

Suspect Hemagglutination Reactions Using Crude vs. Purified Toxoid

	Change in Endpoint Tube	HU/ml*
1. A.G. 0.06 HU/ml		
Crude Ag		0.06
Pure Ag	-1	0.03
2. Y.M. 0.12 HU/ml		
Crude Ag		0.12
Pure Ag	0	0.12
3. Standard 5 AU/ml		
Crude Ag		5.0
Pure Ag	-4	0.3
4. TIG 5 AU/ml		
Crude Ag		5.0
Pure Ag	-3	0.6

* Titer with pure Ag based on Standard with crude Ag.

(2) Comparison of results in man and titrations in animals: Last year we reported the relatively good agreement between the G.M. titers at 28 days observed in man, in the 1966 Cali study, and the International Unit Values determined in mice for Toxoids A, B, C and D by Dr. J. D. van Ramshorst of Utrecht. However, the relative potencies of B and C in mice and men were reversed. Dr. van Ramshorst, reasoning from the fact that adsorption of toxoids enhances the response in mice much more than in guinea pigs, put the 4 preparations on test in 300 guinea pigs. Both by antitoxin titration and by challenge, the guinea pig tests showed B as a better antigen than C -- in agreement with man (see Table 6).

The protection achieved in the prevention of neonatal tetanus, in Dr. K. W. Newell's Guachené study (6) with either 2 or 3 doses of adsorbed tetanus toxoid was so striking, and the 5 year antitoxin titers in a sample of the vaccinees were so well sustained, that it was of interest to test -- in 1967 -- the toxoid used in this study in 1961-63. It had 42 International Units -- less than A (54 I.U.), B (68 I.U.), C (117 I.U.) or D (350 I.U.). Yet it was evidently excellent in potency. Further comparisons of this type are already underway here and in Utrecht.

2. Prevalence and Maintenance of Immunity

a. Tetanus antitoxin levels at varying intervals after the last toxoid injection. The study in collaboration with Dr. T. C. Peebles, mentioned last year, is now completed. One hundred and eighty-two tetanus antitoxin titers were measured in 147 pediatric patients who had received tetanus toxoid immunization according to current practice. No individual who had received 4 or more injections failed to exhibit the accepted protective level of 0.01 AU/ml. Protection after 4 or more injections was calculated, by extrapolation, to extend about 10 years from the last injection with 99.9% confidence limits. Thus it appears that the emergency or "wound" booster may be unnecessary in well immunized subjects. The manuscript is in preparation for publication.

b. Growth and Development Study. Analysis of the data mentioned last year did not reveal any clear-cut correlation with pre-school illness rate, pre-school growth rate or other accessible comparison factors.

c. Reactors to tetanus toxoid. The findings to date were published in the JAMA, October 2, 1967 (see 'Publications', below). It remains to be determined whether different types of reaction can be correlated with the presence or level of IgG vs. IgA antitetanus antibodies. Shortage of staff and space have prevented the initiation of this phase of the study.

d. Long-term immunity. Statistical analysis of the titers of the serum specimens from the Guachené study on prevention of neonatal tetanus (see above, and last year's report) was completed by Dr. Newell's staff at WHO, Geneva. A consistent inverse correlation was found between age level and geometric mean tetanus antitoxin titer.

Table 6

Relative potency of Cali preparations in mouse, guinea pig and man

		Mouse	G.P.	Man				
Preparation	Day	21	28	14	21	28	56	*375
A Lo-Lo		1	1	1	1	1	1	1
B Hi-T, Lo-A		1.26	2.26	1.50	1.77	2.24	2.27	5.18
C Lo-T, Hi-A		2.17	1.72	1.17	1.65	2.10	2.88	2.14
D Hi-Hi		6.48	5.68	1.78	5.38	7.63	4.85	7.14

* A second injection was given on Day 28 or 56

Hi-T: High concentration of tetanus toxoid
Lo-A: Low concentration of aluminum phosphate

Also, longer intervals between injections were correlated with higher titers, and the mean antitoxin levels did not differ significantly irrespective of whether immunization had been commenced 3, 4 or 5 years earlier. This last finding supports the mathematical analysis of residual antitoxin levels published by Gottlieb et al (7) which indicated an essentially zero slope for residual antitoxin after about 3 or 4 years' time since the last injection, and also the results of Peebles et al (manuscript in preparation).

3. Purification and Characterization of Toxins and Toxoids

a. Characterization of slow and fast components of tetanus toxoid. Analysis of proteins contained in tetanus toxin by Klett electrophoresis revealed the presence of two major components with mobilities around 3.2×10^{-5} and 0.5×10^{-5} cm^2/volt/sec. (Fig. 1 a). It was found that the faster component could be separated from the slower component by fractionation on Sephadex G-200. As shown in Figure 2, when proteins collected as Sephadex Fraction II were pooled, concentrated and again analyzed by Klett electrophoresis (Fig. 1 b) they were found to consist primarily of proteins with the faster mobility. Proteins in this fraction contain most of the in-vivo as well as in-vitro activity and demonstrated a mobility in pH 8.6 barbital buffer similar to Pillemer's figure of 2.8×10^{-5} for crystalline tetanus toxin.

The molecular weight of the faster component was estimated by mixing a small amount of Sephadex Fraction II with human serum from a non-immunized individual and fractionating the serum on Sephadex G-200 (Fig. 3). The elution volume for the center of toxic activity was then compared with those obtained for gamma globulin and albumin in whole human serum, as well as for another Fraction II component which was processed separately from crude toxin on the same column. The molecular weight was estimated using the method described by Andrews (8). As shown in Fig. 4, the molecular weight of the faster moving component of tetanus toxin is in the vicinity of 140,000.

When proteins collected under Sephadex Fraction III (Fig. 2) were pooled, concentrated and examined by Klett electrophoresis, they were found to consist primarily of proteins which migrated slowly (Fig. 3 c). However, this analysis also revealed the presence of a reduced but significant amount of the other known toxic component. Since the electrophoretic mobilities of the two components differed, it was decided to attempt further purification of the slow moving protein component in Sephadex Fraction III, by DEAE chromatography.

Studies with crude tetanus toxin have shown that equilibration of both the toxin and the DEAE with .005 M pH 8 phosphate buffer permits the electrophoretically slower component of the toxin to be eluted while the faster component adheres to the DEAE column (Fig. 1 d). However, highly purified solutions obtained by chromatographing Sephadex Fraction III on DEAE cellulose continue to show evidence of tetanus toxicity. Experiments are presently being conducted to determine if this toxicity may still be due to contamination from proteins of the faster component or is actually related to a smaller species of the toxin molecule.

b. Antigenicity of aggregated diphtheria toxoid. Information received last year from European control authorities indicated that the lysine-stabilized purified fluid diphtheria toxoid developed a few years ago at the Wellcome Research Laboratories in England (9) had extraordinarily high antigenicity. Through the courtesy of Dr. M. Sterne of the W.R.L. a large sample of this toxoid was received. It proved to contain an unusually high proportion of a 'dimer' in the range of 140,000 M.W. as estimated by Sephadex separation (see Fig. 5). The monomer and 'dimer' forms are awaiting potency test, as soon as healthy guinea pigs can be found.

4. Studies on typhoid vaccine

Attempts to reproduce the "immunosuppressive" effect of sodium caseinate (10), or other substances (11, 12) which apparently alter the overall pattern of phagocytosis were continued, using typhoid vaccine and challenge with virulent organisms. Caseinate was given 1-2 or 4-5 days before vaccine and both were given either i.p. or s.c. Nine experimental groups to date, employing 1050 mice, have shown that the phenomenon may be complicated, in that the effect of the caseinate was not limited to the i.p. route. Furthermore, a biphasic effect was observed, intervals of less than 3 days between caseinate and vaccine leading to the expected suppression of protection whereas longer intervals were associated with enhancement. Some of Morello and Franzl's findings are consistent with these observations. A working hypothesis, based on these and other findings, has been formulated to explain the discordant results obtained in i.p. vs. s.c. injection of vaccine in active mouse protection tests of typhoid vaccine, and is now being tested.

Meanwhile another published paper by Melikova and Lesnjak (13) adds to the growing literature on artefacts that can apparently distort the immunological response to a mouse protection test. As in the case of the potency tests of tetanus toxoids AL6B and C, one is again reminded of the wisdom of David Henderson (formerly of Porton) when confronted with Felix' "proof" (in mice) that alcohol-type typhoid vaccine was superior, whereas field experience with the alcohol vaccine was distressingly unreassuring. "This just confirms my suspicion", said Henderson, "that there is a difference between mouse and man".

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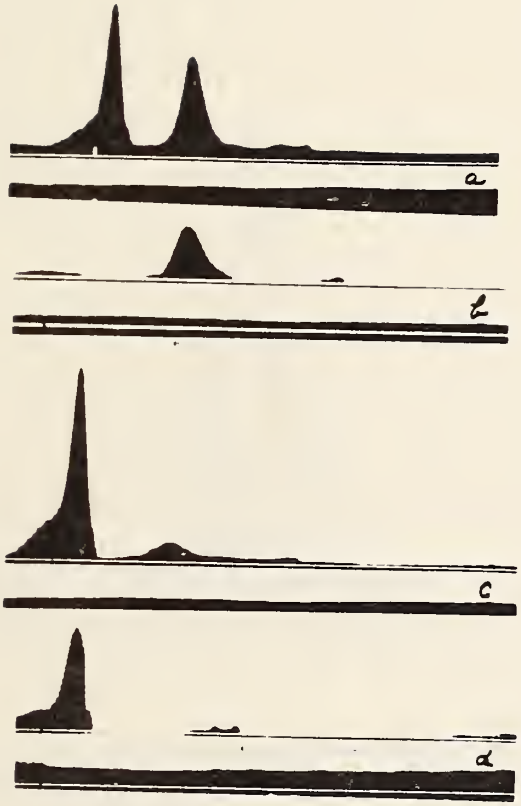

Figure 1. Analysis of proteins contained in tetanus toxin by Klett electrophoresis in 0.1 M barbital, pH 8.6 buffer. (a) Tetanus toxin. (b) Tetanus toxin, Sephadex Fraction II. (c) Tetanus toxin, Sephadex Fraction III. (d) Tetanus toxin, DEAE 0.005 M, pH 8 phosphate Fraction.

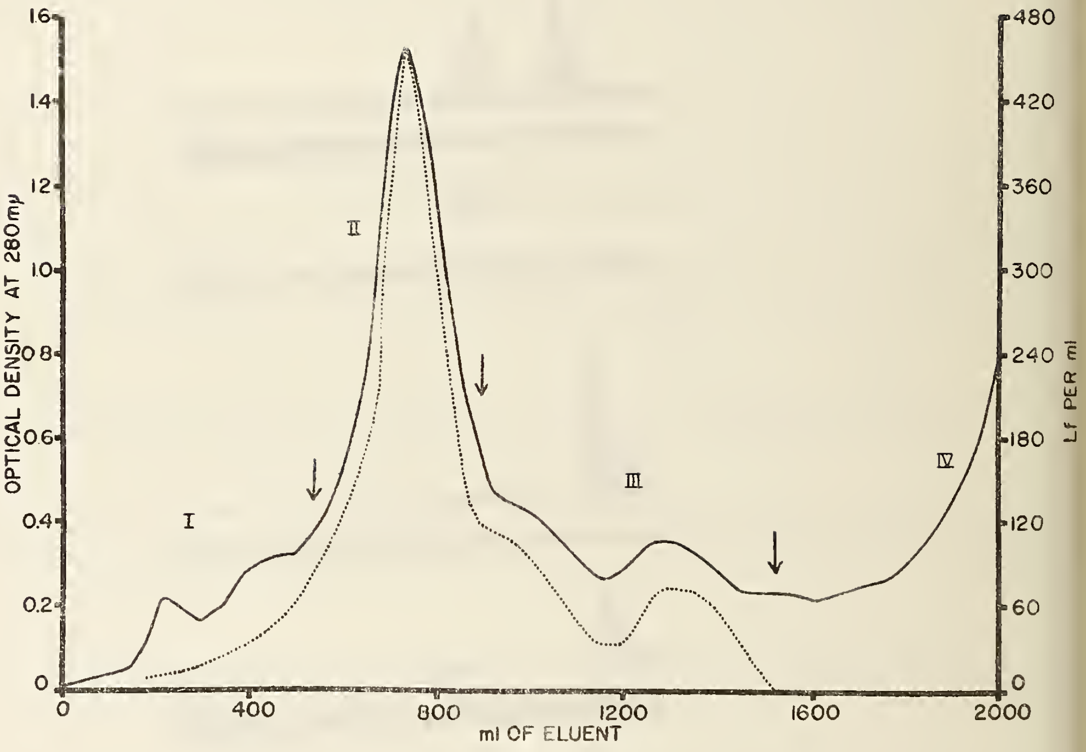

Figure 2. Fractionation of tetanus toxin on Sephadex G-200. Arrows indicate the limits of areas pooled under separate fractions. Solid line, optical density. Dotted line, Lf/ml.

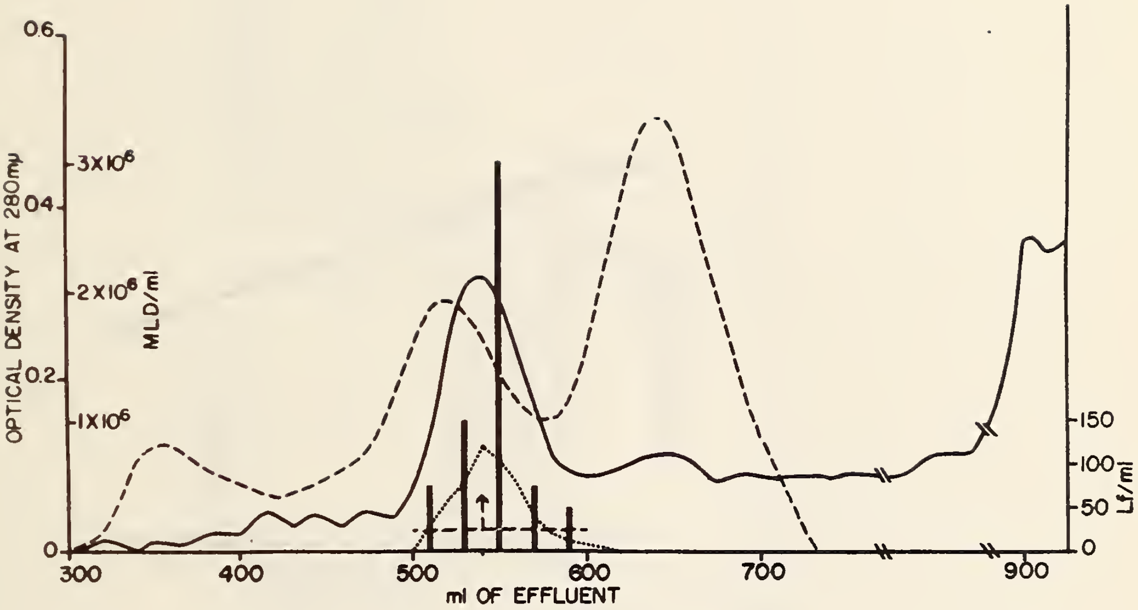

Figure 3. Elution volumes for Sephadex G - 200 fractions for both tetanus toxin and human serum. ———— O.D. at 280 mu, --------- Lf/ml. Bars = MLD/ml for fractions separated from a crude culture filtrate of tetanus toxin. — — — — O.D. at 280 mu for fractions separated from human serum. — —↑— — Represents the center of toxic activity in-vivo for Sephadex Fraction II when a small amount is mixed with human serum and the serum fractionated on Sephadex G - 200.

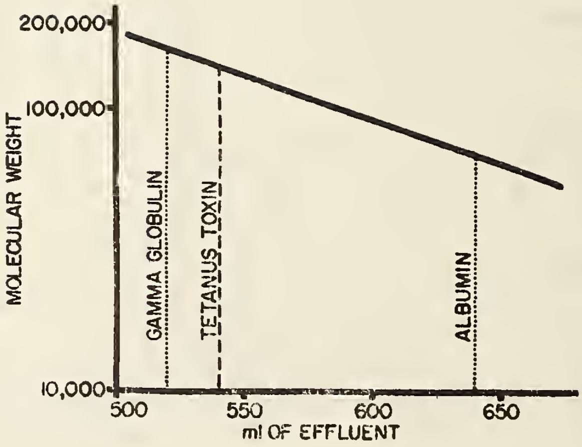

Figure 4. Molecular weight determination of proteins by gel filtration on Sephadex G-200. The elution volumes for the test substances are plotted against their log molecular weight. The molecular weight of the substances are: human gamma globulin 1.6 x 10^5 and human albumin 6.9 x 10^4. Tetanus toxin was calculated to be in the vicinity of 1.4 x 10^5.

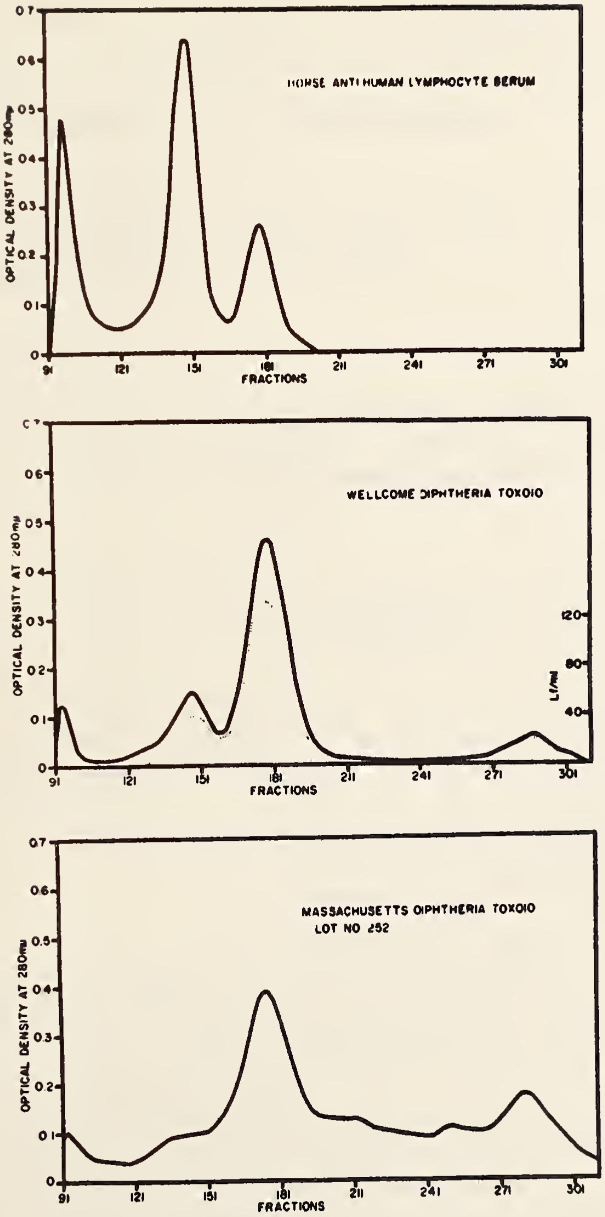

Figure 5. Fractionation of two diphtheria toxoids on a Sephadex G-200 column previously standardized for use in the analysis of horse anti-human lymphocytic serum. A comparison of elution volumes and flocculation patterns indicated that the Wellcome toxoid contained a well defined toxoid dimer in the second fraction.

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13. ABSTRACT

The purpose of these studies is to define the pattern of the immune response in order to improve the efficacy of immunization procedures and to prepare and characterize more highly purified antigens and combinations of antigens, so to facilitate dosage reduction and minimize reactions. (U)

This year, serum samples from the 56-day and 375-day bleedings of volunteers inoculated in 1966 with 4 different tetanus toxoids were titrated for antitoxin. One-half got 2nd injection of toxoid on Day 28, the other half on Day 56. After one injection, Day 56 titers were 50-100% higher than at Day 28 except in the maximal-response group. 56-day levels after 2 injections of toxoid were 12-36 times higher than after 1 injection. Differences in level appeared more closely correlated with [Antigen] than with [AlPO$_4$]. 62 of 63 individuals tested at 1 year (after 2 injections) still carried protective titers. The level was related more to the 1st injection than to the 2nd. A group was started on one injection of the best of the 4 toxoids, to determine the long term protection afforded during 1 year. Studies on passive tetanus hemagglutination titers found in the absence of toxin neutralizing antibody showed they were non-specific, and apparently were due to an unidentified serum factor. Calculations from 182 tetanus antitoxin levels in well immunized children indicated that after 4 doses of toxoid only 1 child in 1000 would have a level below the threshold of protection in the following 10 years. The slow and fast components of tetanus toxin were further characterized. A hypothesis which may explain some of the discrepancies in active mouse protection tests of typhoid vaccines is now being tested.

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ANTIBODY STRUCTURE AND FUNCTION

ANNUAL PROGRESS REPORT

by

Herman N. Eisen, M.D.

(For the period 1 March, 1967 to 30 June, 1968)

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4. Natural antibodies in man with specificity for simple haptens
5. γM anti-DNP antibodies in rabbit, horse, and chicken
6. 7S Chicken anti-DNP antibodies
7. Publications, January 1967 - June 1968.

NOTE

In conducting the research described in this report, the investigators adhered to the "principles of laboratory animal care as established by the National Society for Medical Research."

ABSTRACT

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Annual Report to the Commission on Immunization of the Armed Forces
Epidemiological Board

With 2,4-dinitrophenyl (DNP)-L-lysine as a spectral probe, myeloma sera were screened by a rapid procedure for indications of immunoglobulin binding of DNP-haptens. Of approximately 200 human and 200 mouse myeloma sera examined, 4 active proteins were identified, purified, and studied in varying detail. The one active human protein was $\gamma 2\ \lambda 2$; the 3 active mouse proteins were $\alpha 2$ k2. The affinity for ε-DNP-L-lysine was different for each one, the intrinsic association constants being (in liters/mole at 4°): 2×10^{7}, 3×10^{5}, 6×10^{4}, and 2×10^{4}. The two most extensively studied (K's = 2×10^{7} and 2×10^{4} M^{-1}) were homogeneous with respect to affinity for DNP-lysine. The 2 with highest affinity (K's = 2×10^{7} and 3×10^{5} M^{-1}) precipitated specifically with DNP- and TNP-proteins -- e.g., they did not precipitate the unsubstituted proteins or with conjugates in which the substituents were pipsyl, tosyl, and dansyl, and the specific precipitation was inhibited or reversed with univalent DNP-ligands. The 2 proteins with lowest affinity did not precipitate with DNP- or TNP-proteins.

Corresponding to the unexpectedly high frequency of anti-DNP sites among myeloma proteins, it was possible to isolate from pooled, normal, human IgG and from the IgG fraction of 4 unselected, non-immunized humans a small proportion of the total molecules (about 0.1%) with distinct affinity for the DNP group. These "natural" antibodies to an "unnatural" determinant were recognized and isolated by virtue of their retention on DNP-aminoethyl-cellulose columns, from which they could be specifically eluted by DNP-glycine.

Studies of induced anti-DNP antibodies showed that γM anti-DNP antibodies are made as different proportions of total anti-DNP antibody in different species -- 2% in rabbits, 20% in chickens, and 9% in a horse. The γM anti-DNP antibodies in all 3 species had low affinity (5×10^{4} to 5×10^{5} M^{-1} for ε-DNP-L-lysine at 4°), were heterogeneous with respect to affinity, and had 5 combining sites/900,000 molecular weight. Unlike antibodies of the γG class, their average affinity did not change with time after immunization (at least for 4 months), and did not increase after a booster injection.

Chicken 7S anti-DNP antibodies were obtained in sufficient abundance to be examined spectrally: They brought about the same change in absorption spectrum of bound DNP-ligands that were previously described in a variety of mammalian anti-DNP antibodies; hence, avian and mammalian antibodies of the same specificity must have very similar combining sites, despite the other large differences in their structure (e.g., unlike mammalian antibodies, some of the chicken anti-DNP antibodies only precipitate with DNP-proteins in 1.5 M NaCl, but not in 0.15 M NaCl).

INTRODUCTION

Anti-DNP molecules can probably belong to any of the known immunoglobulin classes; e.g., they probably can have any of the known types of heavy and light chains. The anti-DNP antibodies that belong to the γG class are also highly diversified in structure as revealed by large differences in combining affinity for the DNP group as well as by differences in extinction coefficients, optical rotatory dispersion spectra, and even in amino acid composition (e.g., refs. 8 and 9). The great diversity among the population of antibody molecules of one specificity, made in one individual animal, raises two broad problems:

1) How can one obtain homogeneous antibodies in sufficient quantity to carry out the analytical measures required for establishing directly the structural basis for antibody specificity, and for the variation in structure that account for, or at least correlate with, variations in antibody combining sites as revealed by differences in affinity?

2) How many different homogeneous sets of antibody molecules of a given specificity are made in an individual animal? We do not know, for example, whether one individual can make 10 or 1000 or more different anti-DNP molecules of the γG-immunoglobulin class. If the larger number is correct, then the total number of different immunoglobulins a given individual can make is, perhaps, about a 1000 times greater then the enormous number he must obviously make to be able to account for the capacity to form antibodies to almost any of the thousands of different organic chemicals known to exist, which do not cross react, as far as we know, with the thousands of different proteins to which the same individual can also synthesize specific antibody molecules.

In trying to approach these questions, we set out last year to exploit the facts that: 1) the myeloma protein made by a given plasma cell tumor is homogeneous in structure; 2) each myeloma protein, made by a different tumor, is essentially unique (i.e., identical ones would be rare); 3) there are potentially thousands of myeloma proteins that can be screened for antibody activity; 4) myeloma proteins have all of the gross structural properties of authentic antibody molecules and should therefore have distinct combining sites that make each one of these homogeneous proteins specific for some particular antigenic group.

As is summarized below, we have succeeded in developing a rapid screening procedure, applicable to unfractionated serum, which permitted us to identify one human G-myeloma protein with anti-DNP activity. By then extending the work to mouse myeloma proteins, we have, in collaboration with Dr. Michael Potter of the National Institutes of Health, identified 5 other anti-DNP and/or anti-2,4,6-trinitrophenyl (TNP) A-myeloma proteins, 3 of which have been studied in some detail.

Screening Procedure:

Because it seemed at the outset that the probability was very low for finding a myeloma protein that combines specifically with our model determinant -- the 2,4-dinitrophenyl group -- it was anticipated that many hundreds of these proteins would have to be screened. Accordingly, the screening procedure was designed for unfractionated sera, and we exploited the spectral shift undergone by DNP-ligands when bound specifically by an antibody. Briefly stated, the assay consisted of mixing myeloma serum with ε-DNP-L-lysine and determining the difference spectrum between the mixture and the same concentrations of serum and of hapten in separate tandem cells. These cells (and special cell-holders) wer constructed for use in the double-beam Cary 14 spectrophotometer, with automatic scanning and recording of difference spectra over the wavelengths of particular interest (360-500 mμ) (refs. 1 and 5).

A Human G-myeloma Protein with Anti-DNP Activity.

Of approximately 200 human myeloma sera examined, one gave a positive spectral shift. The myeloma protein isolated from the serum (called BRY, after the patient's name) had the following characteristics:

1) It had λ light chains (no kappa detectable), belonged to the γG-1 subclass, and GM-type was $a^- b^-$ (whereas the whole serum immunoglobulin mixture was $a^+ b^+$).

2) As measured by equilibrium dialysis with ε-DNP-L-lysine, there were two combining sites per myeloma molecule of assumed 145,000 molecular weight.

3) The protein's combining sites were homogeneous in affinity for DNP-lysine.

4) The combining sites were located in the Fab fragment, whereas Fc were inactive.

5) The combining sites were specific. For example, they were only 1/10 as active against 2,4,6-TNP-L-lysine and did not react detectably with ε-2,6-DNP-L-lysine. They also did not bind detectably mononitrophenyls, and even distinguished sharply between 2,4-DNP-L-lysine and 2,4-DNP-D-lysine, which was bound very much less well.

The exquisite specificity and low affinity are characteristic of the anti-DNP antibodies made early in the conventional immune response to DNP protein (ref. 3 and our earlier publications). Indeed, BRY resembled closely these "early" antibodies, except that it seemed entirely homogeneous.

These results are described more fully in reference 5.

Mouse A-Myeloma Proteins with Anti-DNP and Anti-TNP Activity.

By means of the screening procedure outlined above about 200 mouse myeloma proteins were screened, using sera from tumor-bearing Balb/C mice provided by Dr. Michael Potter (of the National Institutes of Health), with whom this work was carried out as a collaborative study. Five proteins were found to bind ε-DNP-lysine and/or t-TNP-L-lysine: one reacted only with DNP, 2 reacted only with TNP, and 2 reacted with both. One of the proteins reacting with both DNP and TNP (MOPC-315) has been studied in considerable detail. The following paragraphs describe the principle findings with this protein.

"Natural" Human Antibodies to an "Unnatural" Antigen.

The finding of several myeloma proteins with anti-DNP activity raised the possibility that in normal non-immunized individuals there might be some immunoglobulins whose combining sites can react with the DNP group. In order to search for such molecules, solid immunoadsorbants were prepared by attaching DNP groups covalently to aminoethyl cellulose (DNP-AE-cellulose). Virtually all amino groups were substituted in order to eliminate ion exchange capacity. Several grams of purified human IgG from pooled human sera were passed through a column of DNP-AE-cellulose and over 99% appeared in the pass-through fraction (called fraction A). About 0.1% was then eluted from the column with CBZ-glycine (fraction B). A third fraction amounting to 0.2 - 0.4% was finally eluted using DNP-glycine (fraction C). When the last fraction (C) was rerun on the same type of column, but with smaller amounts of adsorbant, about half the material appeared in the pass-through, nothing was eluted with CBZ-glycine, and the remaining half was eluted with DNP-glycine (Fraction C2). When fraction C2 was rerun several times further on the same type of column, none of it appeared in the pass-through or after CBZ-glycine elution, but it was all recovered by elution with DNP-glycine. Fraction C2, therefore, behaved as a "natural" antibody to DNP.

Thus, about 0.1% of the IgG molecules in pooled normal human IgG could be isolated by virtue of their selective binding to DNP-amino-cellulose columns followed by selective elution from these columns with DNP-glycine.

Since it was possible that one of the persons contributing to the pooled IgG had been exposed to DNP immunogens, four units of unselected human plasma, discarded from the local blood bank, were examined. From each unit, obtained from a different person, the IgG fraction was isolated by ammonium sulfate precipitation and DEAE-cellulose chromatography; and each individual's IgG was subjected to the DNP-AE-cellulose procedure described above. From the IgG of each of these unselected individuals there was again isolable a fraction, amounting to about 0.1%, with selective reactivity for the DNP-group, as shown by retention on the DNP-adsorbant and elution with DNP-glycine. These "natural" antibodies with DNP-specificity bound ^{3}H-ε-DNP-L-lysine in equilibrium dialysis, and the bound hapten underwent the same shift in absorption spectrum brought about by conventionally induced anti-DNP antibodies.

γM Anti-DNP Antibodies from Rabbit, Horse, and Chicken.

These antibodies were of interest because of the following questions:

1) Can γM molecules exhibit anti-DNP activity? If so, can they display the same broad range of affinity as γG anti-DNP molecules. (10^4 - more than 10^8)? If they form high affinity anti-DNP molecules, it should be possible to exploit this property in trying to answer the "γM paradox" -- i.e., the finding in Pressman's laboratory (Onouè et al) that rabbit γM antibodies to the benzenearsonate group had 5 sites/900,000 molecular weight, whereas 10 were expected on the basis of the γG molecule with its well established bivalency. Another question was whether the γM antibodies made with increasing time after immunization showed the same increase in affinity as is characteristic of γG antibodies.

The following conclusions were drawn after exploration of various DNP immunogens, immunizations schedule, adjuvants, species, and methods of purification.

1) After immunization with one injection of DNP-BγG or DNP-Hemocyanin in Freunds adjuvant, anti-DNP antibodies of the γM class could be isolated from bleedings obtained from 4-5 days up to several months later. The γM antibodies were isolable by immunoadsorption on DNP-HSA cellulose following by elution with DNP-glycine and chromatography of the eluate on DEAE-cellulose. γM levels were surprisingly constant for many weeks after this type of immunization, the level varying with species; e.g., the proportion of total anti-DNP antibody which was γM was about 2% in rabbits, 9% in a horse, and 20% in chickens.

Examined by equilibrium dialysis with ^{3}H-DNP-L-lysine, the γM antibodies from all 3 species were approximately the same: all were heterogeneous with respect to affinity, the average association constant was low, about 5×10^4 to 5×10^5 M^{-1}, binding was essentially athermal ($\Delta H \simeq 0$), and there were 5 sites/ 900,000 molecular weight, in confirmation of the measurement made by Onoue _et al_ with anti-benzenearsonate.

Unlike γG antibodies, there was no change in affinity with time after immunization, at least up to 4 months. There was also no increase in affinity of the antibodies produced after a second injection as compared with those formed after the first injection.

7S Chicken Antibodies to the DNP Group.

In the course of preparing chicken γM antibodies to DNP, large amounts of 7S antibodies of the same specificity were isolated. These were studied from two points of view.

1) Since all the anti-DNP and anti-TNP antibodies from various mammalian species (rabbit, goat, horse, guinea pig; human and mouse myeloma proteins) showed a characteristic spectral shift of absorbed DNP-hapten, the question was raised whether the antibodies from a phyletically distant species, such as a bird, would have a similar combining site and produce the same change in absorption spectrum of bound hapten. The answer was clear: when DNP-lysine was bound by chicken anti-DNP antibodies, the bound hapten underwent the same change in absorption spectrum as when bound specifically by mammalian antibodies.

2) There are two kinds of chicken 7S antibodies, distinguishable by immunoelectrophoresis. One of them precipitates with antigen as do mammalian antibodies, i.e., in 0.15 M NaCl; but the other does not precipitate with antigen

unless the salt concentration is increased to about 1.5 M. The question arises therefore, whether the high-salt requiring antibody has a change in affinity with salt concentration. By equilibrium dialysis and by fluorescence quenching studies, binding was found to be the same in both 1.5 M and 1.5 M NaCl. Hence, its non-precipitability with DNP-protein in 0.15 M NaCl was not due to a change in the combining site. An incidental finding was that the pepsin fragment (5S bivalent) prepared from this high-salt requiring antibody exhibited the same dependency on salt for precipitation as did the intact molecule. Hence, the unusual structural features of this chicken antibody, which accounts for high-salt dependency of its precipitability, reside in the Fab rather than in the Fc domain.

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2) Natural human anti-DNP immunoglobulins: Dr. Fuad S. Farah, on sabbatical leave from the American University of Beirut, Lebanon.

3) γM anti-DNP antibodies: Dr. Edward W. Voss, Jr.

4) Chicken 7S antibodies: Dr. Edward W. Voss, Jr.

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STUDIES ON THE IMMUNIZATION OF MAN AGAINST PLAGUE (U)

Annual Progress Report No. 18

by

K. F. Meyer, M.D., Director Emeritus
G. W. Hooper Foundation

(For the period 1 March 1967 to 30 June 1968)

U.S. Army Medical Research and Development Command
Department of the Army
Washington, D.C. 20315

Annual Report to the Commission on Immunization
of the Armed Forces Epidemiological Board

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SUMMARY

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(2) Studies on the Immunization of Man Against Plague

(3) K. F. Meyer, M.D., Director Emeritus, Principal Investigator

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Annual Report No. 18 to the Commission on Immunization of the Armed Forces Epidemiological Board

In a clinical trial on 25 human subjects each inoculated intramuscularly with 1 ml of dead U.S.P. plague vaccine (E medium) in accordance with the newly issued directions for military use (May 1967), the vaccine caused mild local and systemic reactions. The basic inoculations reduced the MPI (Mouse Protection Indices) to below 10 in 16% and below 5 in 4% by the 28th day; thus a single inoculation of dead vaccine confers little or no immunity. However, a small booster inoculation of 0.2 ml brings the MPI to below 10 in 65% and below 5 in 26% of human subjects.

An experimental alum-adsorbed dead plague vaccine stored for nine months at ±4^{o}C apparently deteriorated; it proved inferior to the U.S.P. Cutter vaccine in this respect. Until a stable preparation has been manufactured, the U.S.P. vaccine remains the only plague prophylactic available for mass inoculations of Armed Forces personnel.

The U.S.P. dead plague vaccine administered in two inoculations three months apart protected six of nine (66%) Hanuman langurs (Presbytis entellus) with significant mouse protection indices. They survived plague infections which proved fatal to non-inoculated controls in five to six days. None of the langurs with an MPI above 10 survived. This simian genus is definitely more susceptible and more uniform in

its reaction to plague than Macaca mulatta. The langurs' response to immunization is heterogeneous. Some lack an effective immunity mechanism, which is indirectly reflected by low MPI, and succumb to subacute, mitigated plague infections.

A clinical trial with a sample of freeze-dried living attenuated P. pestis EV 76 (WR-S) vaccine in large doses of 53,500,000 viable organisms produced fleeting local erythemas and some palpable lymph nodes, but no rise in temperature or general malaise. The antibody response after inoculation was disappointing: mouse protection antibodies before and after inoculation remained essentially the same. The vaccine does not sensitize. No booster dose effects were elicited with either live or dead plague vaccines.

A clinical trial on 25 men each inoculated subcutaneously with 420,000,000 viable freeze-dried living attenuated P. pestis EV (WR-S) induced pronounced erythema and some induration lasting for 96 hours in 23 (92%) and severe local reactions in two. All developed lymphadenopathies; six (24%) had fever, and seven (28%) disclosed a slight rise in WBC. Hard, sometimes tender areas or small indurated nodules below the needle puncture persisted for two weeks or longer. These reactions were more severe than those produced by U.S.P. plague vaccines and were considered distinctly unpleasant by the subjects. The antibody response was of the same magnitude as that recorded for the sera of men inoculated with dead plague bacilli. Only two sera yielded mouse protection indices of 10 and below. U.S.P. plague vaccine and Fraction 1, but not the living plague vaccine in small dosage, proved effective as booster inocula in the inadequately sensitized human subjects. Intradermal inoculations of dead plague vaccine, even with the most careful intracutaneous injection technique, induced persisting indurated nodules.

Comparative vaccination and resistance tests on small groups of Hanuman langurs confirmed previous observations. The EV (WR-S) vaccine in a dosage of 53,000,000 freeze-dried viable plague bacilli protected five out of six langurs against fatal but not local plague infection. With two exceptions, immunity was reflected by the presence of significant protective antibodies (1.0 - 4.0) in the peripheral blood serum. One langur with an MPI of 14 survived, while another with an index of 1.0 developed plague bacteremia and succumbed to an overwhelming secondary enteric infection. On recovery from plague, the vaccinated langurs had much higher HA and CF antibodies by the 36th day than they showed after receiving only the vaccine.

Antibody response was of a high order in Presbytis entellus inoculated subcutaneously with 420,000,000 or 42,000,000 freeze-dried viable P. pestis EV (WR-S) in a total suspension of 200 and 2 billion plague bacilli respectively. There were even complement-fixing antibodies (1:4 to 1:64) and HA titers as high as 1:32,768. Except in

2 of 16 *Presbytis*, mouse protection indices were all below 5 on the 28th day after vaccination. Since past experience had demonstrated that langurs with high-level antibodies would survive severe challenge infections, it was decided to apply this experimental series to the determination of duration of immunity. The langurs will be divided into two groups and infected with virulent plague. One group will be challenged by the subcutaneous and the other by the aerosol route during the 16th month after vaccination. Some animals will receive a second inoculation of the original 420 million vaccine dose as is practiced in human mass vaccinations in Madagascar.

The susceptibility of the *aethiops* group of the African subgenus *Cercopithecus* to plague was evaluated, since it became increasingly difficult to import adequate numbers of langurs from India, thus restricting the scale of experiments which may ultimately furnish meaningful conclusions. The response of *Cercopithecus aethiops* (imported from Ethiopia, exact subspecies not determined) to subcutaneous plague infection was not uniform, but in general it was similar to that of langurs in individual resistance, course of infection and time of death. Like langurs, individual *Cercopithecus* may succumb to 200 to 200,000 *P. pestis*, or, when they survive, develop significant antibodies. Two late deaths on the 29th day after infection with 206 *P. pestis* were attributed to septicemia mitigated by secondary metastatic pulmonary plague as has been observed in guinea pigs. The pilot susceptibility experiment encouraged the use of subhuman primates belonging to the *aethiops* group of *Cercopithecus* in vaccination and protection tests.

It was assumed that all species of the *aethiops* group of the subgenus *Cercopithecus* would react to plague in the same manner as the species from Ethiopia. Seven well conditioned *Cercopitheci* imported from Kenya were included in an experimental trial to evaluate the immunogenic and protective efficacy of living EV (WR-S) on langurs and human subjects. The subcutaneous inoculations were well tolerated, but the antibody response was negligible even after booster inoculations. The entire group of vaccinated *aethiops* succumbed to acute septicemic plague either before or after the death of the only control animal. Failure to respond to vaccination was surprising, since in a comparable experiment the EV (WR-S) vaccine had conferred good protection on langurs. Subsequent experiments indicated that the *Kenya aethiops* is more susceptible to plague than the Ethiopian species. Negotiations are in progress to repeat this type of experiment on large series of South African *Cercopithecus pygerythrus*.

In the search for an EV 76 isolate with greater invasive potential, persistence and correspondingly enhanced immunogenicity and protective properties, the freeze-dried growth prepared from a culture received from Madagascar in 1951 was passaged through guinea pigs pretreated with $FeSO_4$ (iron sulfate). The isolate from the spleen of the sixth guinea pig passage, designated EV 76 51f and consisting of typical smooth *P. pestis* colonies, was readily isolated by phage with the biochemical reactions of the oceanic race and the determinant characters F_1+, V&W+, Pu+ and P-, and proved nonpathogenic for mice upon subcutaneous inoculation with 3,000-6,000, and for guinea pigs with 6.7 billion. The protective efficacy expressed as PD_{50} was 5 viable organisms for guinea pigs and 1,100 for mice. Intraperitoneal inoculations in dosages of 10 billion viable EV 51f induced toxic death within 24 to 72 hours. Subcutaneous, cutaneous and intracutaneous inoculations with billion dosages of the isolate produced typical granulomas in the regional lymph nodes, the spleen and occasionally the liver. According to generally accepted standards and pathogenicity and protection tests on guinea pigs, the EV 51f isolate was considered "safe". However, when it was tested in vaccination trials on *aethiops* sp. groups of subhuman primates of the genus *Cercopithecus*, surprisingly it produced illness and death. In a trial on *Cercopitheci of Ethiopian origin*, subcutaneous vaccination with 160 million viable EV 51f in a group of four animals caused acute fatal plague septicemia on the fifth day. One of four *Cercopitheci* vaccinated with 1 million met the same fate; extended illness and emaciation, with death on the 18th day, were observed in one of four monkeys vaccinated with 16 million organisms. At autopsy, typical granulomas were present in the liver and enlarged spleen, which yielded the vaccine strain in pure culture. All survivors developed antibodies with high titers. The low mouse protective indices were particularly noteworthy. With no local reactions, the three remaining animals of the vaccine trial survived a severe plague infection which was fatal within four to six days to *Cercopitheci* inoculated with a placebo.

In a second vaccination trial on 20 *aethiops* sp. of the genus *Cercopithecus* imported from Kenya, using reconstituted freeze-dried EV 51f vaccine and undertaken for the purpose of studying the clinical course of the vaccination disease more closely, the outcome was startling: Fifty percent of the animals vaccinated subcutaneously with 2,180, 218,000, 2 million or 218 million exhibited local reactions, with fever and rises in WBC, and from the 72nd to 96th hour on, yielded positive *P. pestis* blood cultures, and in 6 to 28 days succumbed with lesions and bacteriologic evidence of plague. Antibody response in the peripheral blood of survivors of the vaccinations was vigorous and particularly pronounced in the animals vaccinated with 218 viable EV 51f. They were not clinically affected except for a transient rise in WBC, and the blood yielded no positive cultures. They were completely protected against subcutaneous plague. Although the trial is not yet complete and the records not completely analyzed, it is concluded (a) that the unidentified subspecies of *Cercopithecus* from *Kenya* is probably one of the most plague-susceptible subhuman primates thus far tested; and (b) that the EV 51f, a descendant of the original EV 76 of Girard and Robic, has considerable residual "virulence" for this simian species.

The following immediate studies are proposed:

(1) Comparative tests to determine the stability of the freeze-dried living EV 51f vaccine at frequent intervals.

(2) Since in order to avoid unpleasant local and systemic reactions, Soviet immunologists recommend the cutaneous or scarification method of plague vaccination with living vaccines, it is advisable to titrate and determine the minimum dose of EV 51f required to stimulate significant mouse protection indices in human subjects.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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I. Immunogenic and protective efficacy of formalin-killed virulent P. pestis U.S.P. plague vaccine (E medium)

Report #17 (pp. 5-10) briefly outlined a pilot protection experiment on Hanuman langurs and a clinical trial with two lots of aqueous suspensions of formalin-killed virulent P. pestis grown on a new culture medium free from undesirable blood-group substances and fully immunogenic in the mouse potency test. One lot (E 34819) failed to protect the three langurs inoculated. The basic inoculation of human subjects stimulated HA, but mouse protective antibodies were elicited in only two men (Nos. 11 and 16), who had previously been vaccinated. An intramuscular booster inoculation (0.2 ml = 400,000,000 P. pestis) raised the MPI below 10 from 0 to 50% and below 5 to 20%. Similarly it proved an efficient booster antigen in men previously inoculated with triple (cholera-plague-typhus) vaccine. The second lot (E 34820), which after basic inoculation stimulated MPI in 17% of the men to a level below 10, following the booster further stimulated the level to 60% below 10 and 25% below 5. Since these levels are slightly superior to those determined for Cutter plague vaccine prepared during the past 20 years, mass production of the U.S.P. plague vaccine (E medium) was recommended and initiated in 1967.

For the purpose of checking the results of mass production and holding the vaccine in bulk, Cutter Laboratories was requested to supply samples for additional protection tests on subhuman primates and clinical trials on human subjects. In January 1967, during the manufacture of Lot K 1668 from concentrate M.O. 35459 (16.4×10^{-9} P. pestis, 0.78% formalin, total nitrogen 2.74 mg/ml = 0.167 mg/10^{-9} organisms), two lots were compounded and supplied in vials.

(A) Aqueous suspension of 2.0×10^{9} ml killed P. pestis. This preparation gave a mouse potency test (Standard 6662) with 4.0 ratio.

(B) Alum-adsorbed plague vaccine CC 62357A (M.O. 35588) with the following composition: 2.0×10^{9} ml plague bacilli, total nitrogen 0.334 mg/ml, phenol 0.52%, formalin 0.030%, Al_2O_3 0.069%, yielded a mouse potency ratio of 10.

Both preparations were held for nine months to measure the shelf stability and efficacy of the U.S.P. plague vaccine when administered in accordance with the newly issued "Directions for Military Use" (May 1967). The usual standard methods of testing were followed: (1) Groups of Hanuman langurs, small because it was not feasible to import larger numbers from India, were inoculated intramuscularly with the two types of vaccine; and on the same day, (2) 50 human subjects, divided into two groups and under constant medical supervision at the California Medical Facility, Department of Correction, Vacaville, California, received their basic and booster inoculations. Immunogenic efficacy was measured indirectly by serologic tests on peripheral blood serum specimens collected at frequent intervals.

(1) The protective efficacy of U.S.P. plague vaccine in Hanuman langurs (*Presbytis entellus*)

Groups of five thoroughly conditioned langurs were each inoculated intramuscularly with 1 ml of U.S.P. plague vaccine (E medium), while four were treated similarly with alum-adsorbed plague vaccine, experimental lot CC 62357A. The only serologic tests made with their blood sera on the 35th day after basic inoculation demonstrated HA antibodies in titers varying from 1:16 to 1:128 (no CF) and Mouse Protection Indices (average 11) of which three were in the group of 10 or below. Eighty-six days after basic inoculation, each group received intramuscular booster doses of 400 million *P. pestis* of the respective vaccines. At that time, one primate not previously vaccinated was inoculated with 0.2 ml (or 400 million) of the aqueous vaccine. The significant antibody titers after booster at time of challenge listed in Table 1 indicate that the immune response expressed in HA was vigorous in four and moderate in five, but that MPI below 10 were present in seven, of which five had MPI of 5 or less. The langur (SE 280) given only the 0.2 ml booster of U.S.P. vaccine had no significant antibodies. On the 40th day after booster (or 125th day after primary inoculation), both groups, together with three non-inoculated langurs as controls, were subcutaneously infected with 131,000 virulent *P. pestis* 195/P. The controls succumbed to septicemic plague within five to six days; one langur (SE 255), inoculated with the U.S.P. plague vaccine and properly boostered, failed to develop a significant MPI (18) and died at the same time as the controls. Three of five langurs in Group A survived with MPI below 10 (1, 3, and 5) prior to challenge. One with an index of 13 died on the 16th day with lesions of secondary metastatic pulmonary plague. Another fatality, (SE 280, with an MPI of 15) had been inoculated with a small dose (0.2 ml) of U.S.P. plague vaccine at the time of booster. Langur SE 271 in Group B, inoculated with the alum-adsorbed vaccine, had an MPI of 3, became less active, took food irregularly, and died unexpectedly. All three fatalities in Group A had abscesses at the site of infection and enlarged axillary lymph nodes. At autopsy the principal lesions in the lungs represented progressive stages of pneumonia. The langur that died on the 16th day presented discrete yellowish nodules throughout the voluminous congested lobes on both sides, or conglomerate masses surrounded by purplish areas of consolidation similar to the embolic metastatic pulmonary plague commonly encountered in guinea pigs surviving for six days or longer. An extremely advanced state of consolidation, yellow and suppurating, in the left upper, middle, and lower lobes was discovered upon autopsy of SE 271, which unexpectedly died on the 35th day after infection. The right voluminous lobe exhibited varying grades of congestion. Langur SE 280 began to cough on the 15th day after infection. At autopsy, the left and right lower lobes, covered with fibrinous adhesions to the thoracic wall, were in a state of complete consolidation except for the lower margins. Necrotic metastatic nodules were distinctly visible in the middle lobes. The lymph

TABLE 1

Antibody Response in langurs (*Presbytis entellus*) before and after plague infection. Intramuscular inoculations with (A) Dead vaccine and (B) Alum-adsorbed U.S.P. plague vaccine (Experimental lot).

Primary inoculation: 1 ml
Booster inoculation: 0.2 ml (86th day)
Challenge infection: 40th day after booster

	Antibody Titers at Time of Challenge			Reaction to Plague Infection				Antibody Titers After Recovery from Plague			
								28th day		66th day	
	HA	CF	MPI	Blood Culture	Day of Death	S/T	Pathology	HA	CF	HA	CF
A. SE 242	2,048	0	1	-	-		-	6,500	32	2,048	4
SE 254	4,096	0	3	-	-		-	32,000	16	16,384	4
SE 255	64	0	18	+	6th		Septicemia	-	-	-	-
SE 279	128	0	13	+	16th	3/5	Metastatic Pulmonary Plague	-	-	-	-
SE 258	512	0	5	-	-		-	131,000	64	16,384	4
/SE 280 (C-0.2)/	0	0	15	-	21st		Metastatic Pulmonary Plague	-	-	-	-
B. SE 266	4,096	0	00	-	-		-	65,000	32	8,192	0
SE 265	128	0	7	-	-	3/4	-	1,024	8	2,048	2
SE 271	256	0	13	-	35th		Metastatic Pulmonary Plague	-	-	-	-
SE 272	4,096	0	15	-	-		57th axillary abscess ++	not bled		32,768	16
Control SE 314			16	+	5th		Septicemia				
SE 292			16	+	6th	0/3	Septicemia				
SE 286			18	+	5th		Septicemia				

S/T = Survivors/Total

nodes of the thoracic cavities--bronchial and mediastinal--appeared as secondary buboes. Aside from the lungs, the heartblood, spleen and axillary lymph nodes yielded pure cultures of virulent P. pestis. These very interesting histologic lesions are the subject of a cooperative study by Captain Robert E. Kanich, Armed Forces Institute of Pathology.

The protracted disease, seen for the first time in langurs and designated as "chronic" plague in the past, resembles "mitigated" or "relapsing" plague in partly immune rodents. Undoubtedly the abscess in the axillary lymph nodes, which yielded high-virulent P. pestis on the 57th day, was perhaps a mere "peripheral" lesion in langur SE 272. No special attempt was made to determine whether the primate also had visceral lesions. X-ray facilities for examining plague-infected animals were not available and stethoscope examinations were inconclusive. Streptomycin in two inoculations of 1 mg each promptly cured the axillary lesions.

Despite the small number of langurs available to measure the protective efficacy of the U.S.P. plague vaccine, some conclusions may be stated:

(a) The vaccine, administered in two inoculations three months apart, protected 6/9 or 66% of the simian subjects with significant serum mouse protection indices. These animals survived severe plague infections which proved fatal to non-inoculated controls in five to six days.

(b) The alum-adsorbed vaccine was similarly protective but was not superior to the standard preparation without the synergist.

(c) It was noted that all langurs surviving plague exhibited either no reactions or well defined local indurations at the site of infection.

(d) Resistance to plague was indirectly reflected by the MPI which were below 10 (five below 5). None of the langurs with an MPI above 10 survived; in fact, SE 255, with an MPI of 18, succumbed at the same time as the controls; and SE 279, with an index of 13, died from metastatic pneumonia. As anticipated, a single 0.2 ml inoculation of the U.S.P. vaccine failed to stimulate HA and CF antibodies but reduced the MPI to 15 and delayed death to 21 days. The late death of SE 271, with an MPI of 3, remains unexplained. These correlations confirm results of the protection tests conducted on two species of langurs (P. entellus and P. cristatus) inoculated with the "Exotic Package vaccine" (Annual Report #16, pp. 12 and 13). Only langurs with an average MPI of 7 or below 5 survived plague infections induced with 8,100 to 81,000 virulent P. pestis.

(e) Various protection experiments with small groups of langurs indicate that this simian genus is definitely more susceptible and slightly more uniform in its reaction to plague than Macaca mulatta. The immunization capacity of members of this genus, however, is heterogenetic. The majority of langurs that survive importation, time-consuming conditioning with prophylactic chemotherapy, and colonization procedures are capable of responding with an effective immunity mechanism indirectly reflected by the low MPI. A few, however, lack this ability and succumb to subacute, mitigated plague infection.

(f) The high-titer antibody response following recovery and persisting for two months, and in particular the appearance of CF, are noteworthy and confirm the suitability of Presbytis entellus as an experimental host for studies of plague.

(g) The dead U.S.P. plague vaccine administered in accordance with directions for military use protects approximately 60% of inoculated langurs.

(2) Clinical trial of U.S.P. plague vaccine on human subjects.

Groups of 25 human subjects were each inoculated intramuscularly with 1 ml of the dead U.S.P. plague (E medium) vaccine preparations. Local and systemic reactions were all mild; lymph nodes were palpable in two men inoculated with the aqueous vaccine and in one subject treated with the alum-adsorbed preparation. The latter also had a slight rise in temperature. Complaints of myalgia and malaise were made by subjects with mild respiratory infections, but by the 72nd hour the reactions had disappeared. Passive hemagglutinins in titers varying from 1:8 to 1:256 were noted on the 8th and 16th day in 15 of 25 men inoculated with the aqueous preparation and 20 of 25 men treated with the alum-adsorbed vaccine. By the 28th day, all but one of the subjects reacted with HA titers of 1:16 to 1:1024. Within three months the titers had declined to lower levels or disappeared in one quarter of the vaccinated. Single booster inoculations of 0.2 ml (400,000,000 formalin-killed P. pestis) of the respective vaccine preparations restored the HA to original levels.

In view of the observations on langurs, in-vivo-determined passive mouse protection indices (MPI) deserve special analysis. The indices below 10, and particularly those below 5, indirectly reflect the state of immunity induced by a plague vaccine. Analysis of the data collected (Tables 2 and 3) in the course of nine periodic examinations over an interval of 140 days and condensed in Table 4, permits the following interpretations and conclusions:

(a) U.S.P. plague vaccine inoculated in a single dose of 1 ml (2,000 million formalin-killed virulent P. pestis) reduced the MPI of 25 human subjects to below 10 in 16% by the 28th day and to below 5

TABLE 2

Local, Systemic and Serologic Reactions Recorded in 25 Human Subjects Inoculated Intramuscularly with Plague Vaccine U.S.P., E Medium (Lot K1668) Aqueous, 1 ml.

Basic immunization: October 10, 1967
Booster inoculation: January 4, 1968, 0.2 ml intramuscularly

		Serologic Response							
		Basic inoculation			Pre-booster		Post-booster		
	Clinical	28th day			84th day		28th day		
Number	Reactions	HA	CF	MPI	HA	CF	HA	CF	MPI
1. Co	00+	128	0	18	0	0	0	0	21
2. Pe	00±	64	0	18	4	0	0	0	7
3. Le	000	16	0	13	0	0	-	-	-
4. Ga	0++	128	0	9	0	0	0	0	17
6. Ba	+0+	1024	0	8	32	0	64	0	7
7. Bo	+0+ L	256	0	18	256	0	128	0	10
8. Ci	00+	128	0	13	16	0	0	0	9
9. Ra	00+	128	0	12	8	0	0	0	7
10. Ro	+0+	64	0	10	64	0	128	0	11
11. Gr	000	32	0	15	8	0	0	0	15
12. Ro	+0+	16	0	16	0	0	2048	0	6
13. Bo	00± F.M.	512	0	11	128	0	1024	0	2
16. Av	00±	512	0	16	16	0	256	0	14
17. Po	00+ L	256	0	12	8	0	1024	0	4
18. Pe	00±	64	0	15	4	0	1024	0	2
19. Mc	00+	128	0	16	64	0	1024	0	4
21. Pe	0+±	128	0	15	4	0	512	0	2
22. Bl	000	128	0	14	16	0	256	0	6
23. Se	000	512	0	12	8	0	2048	0	00
24. Ca	00± F.M.	64	0	4	0	0	0	0	6
25. Ba	+0+	0	0	17	0	0	0	0	16
27. Ri	00±	32	0	16	8	0	0	0	9
28. Ha	00±	128	0	13	64	0	512	0	12
29. Do	000	128	0	15	8	0	0	0	11
30. Hu	00+	64	0	-	-	-	-	-	-
Average:		185.5		13.5	29		432		8.6
MPI 10 and below				16%					65%
MPI 5 and below				4%					26%

L = Lymphadenopathy
F = Fever
M = Myalgia

TABLE 3

Local, Systemic and Serologic Reactions Recorded for 25 Human Subjects Inoculated Intramuscularly with Plague Vaccine Alum-Adsorbed 1 ml. Vaccine Lot: CC62357A Experimental.

Basic immunization: October 10, 1967
Booster inoculation: January 4, 1968, 0.2 ml intramuscularly

		Serologic Response							
		Basic inoculation 28th day			Pre-booster 84th day		Post-booster 28th day		
Number	Clinical Reaction	HA	CF	MPI	HA	CF	HA	CF	MPI
31. Co	0++ M	128	0	11	0	0	-	-	-
32. No	±++	64	0	10	8	0	256	0	10
33. Br	00+	256	0	11	0	0	0	0	12
34. Ka	00± M	128	0	11	4	0	256	0	10
35. Bi	00+	128	0	11	4	0	512	0	10
36. Ev	±00	256	0	13	8	0	0	0	11
37. Ba	00±	256	0	14	16	0	0	0	7
38. Gr	00±	128	0	13	8	0	128	0	12
39. Pi	00+	32	0	13	4	0	256	0	10
40. Fo	00+	128	0	14	4	0	256	0	11
41. Pa	+0±	128	0	8	16	0	64	0	12
42. Ca	00+	128	0	11	128	0	512	0	6
43. Mo	00+	16	0	12	0	0	0	0	11
44. Mo	00+	128	0	12	4	0	64	0	10
46. Ha	+0+ M	128	0	17	128	0	512	0	7
47. Cr	000	128	0	15	4	0	128	0	17
48. Bl	00+	64	0	20	4	0	0	0	18
49. De	0++	64	0	17	32	0	128	0	11
50. St	00+	512	0	-	4	0	-	-	-
52. Ma	00+	512	0	9	4	0	256	0	10
54. Ed	00+	128	0	6	32	0	1024	0	2
55. Mo	00+	128	0	15	0	0	512	0	8
57. Ca	00+ L.F.M.	128	0	14	16	0	256	0	4
58. Pi	00+	256	0	10	128	0	128	0	3
59. Fi	00+	64	0	8	8	0	64	0	7
Average:		160.7		12.2	24.1		230.1		9.5
MPI 10 and below				20%					60%
MPI 5 and below				0%					17%

L = Lymphadenopathy
F = Fever
M = Mylagia

TABLE 4

Serologic Reactions Recorded in 20 Randomly Selected Human Subjects Following Basic Intramuscular Inoculation with U.S.P. Plague Vaccine 1 ml (=2 billion) Followed by Booster 0.2 ml (400 million) Inoculated on Day 60 after Basic Inoculation.

	After Basic Inoculation				After Booster	
	30th day		60th day		30th day	
	HA	MPI	HA	MPI	HA	MPI
1.	32	-	8	17	4	19
2.	128	-	8	13	8	15
3.	512	15	512	15	128	10
4.	256	13	128	16	64	17
5.	128	13	-	20	64	3
6.	256	14	128	18	64	6
7.	R 32,768	2	32	18	-	2
8.	256	17	16	15	4	15
9.	16	15	128	12	128	8
10.	2,048	13	256	18	64	16
11.	R -	3	65,536	4	-	1
12.	256	-	1,024	13	32	13
13.	256	14	64	10	512	9
14.	8	12	64	13	32	10
15.	512	11	1,024	7	512	5
16.	R 16,384	00	16,384	1	32,768	00
17.	1,024	6	128	17	256	6
18.	128	20	512	14	32	7
19.	64	17	64	19	8	14
20.	128	12	64	12	64	13
Average:		12		13		9.4
MPI 10 and below		23%		15%		60%
MPI 5 and below		17%		10%		25%

R = probably revaccination

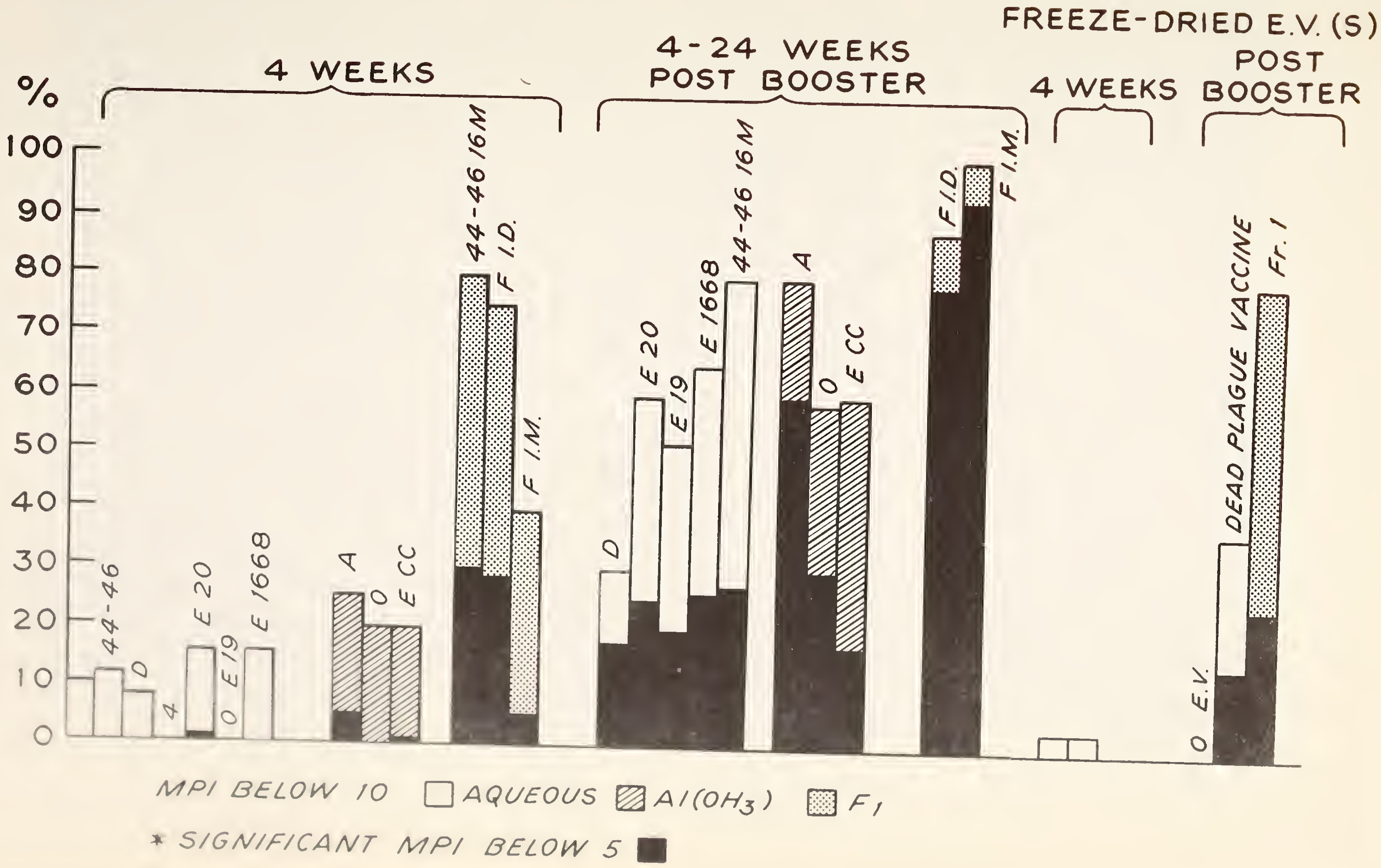
in 4%. Thus a single inoculation of vaccine confers little or no immunity, although the E medium preparation is slightly superior to lots available in 1960 represented in the seventh column of the histogram chart on p. 31 of Annual Report #17. A small booster inoculation of 0.2 ml brought the MPI to below 10 in 65% and below 5 in 26% of the human subjects. The newly manufactured U.S.P. Cutter plague vaccine appears to be slightly superior to that produced in 1967.

It is imperative that the sera of at least 100 to 200 Armed Forces service personnel undergoing prophylactic inoculation be examined in order to verify the results collected in relatively small clinical trials. A detailed review of previously reported trials to raise the MPI above 65% by second booster inoculations at intervals of six months failed to furnish encouraging data. With two exceptions, the second vaccine booster merely recalled protective antibodies to the same levels. Larger doses of dead vaccine were invariably complicated by resultant unpleasant local and systemic reactions with no significant rise in the indices. However, it is noteworthy that inoculations with Fraction 1 antigens for active anti-plague immunization pushed the MPI to 90% below 5 in two clinical trials with 10-16 human subjects (Annual Report #2, 1952, and histogram chart).

(3) Clinical trial using the synergist alum-adsorbed U.S.P. plague vaccine.

The trial to be reported was disappointing. The advantages of synergist antigen vaccines over saline suspension ones was demonstrated in a series of experiments and clinical trials (see Annual Reports #12, 13 and 14). Ever since trials with batches of alum-adsorbed vaccines, and particularly experiences with the cholera-plague-typhus vaccine containing alum, indicated that the immunogenic efficacy of these types of preparations is unstable, studies to evaluate the storage life of alum-adsorbed E-medium-grown P. pestis vaccines were contemplated. Urgent interest in attenuated viable EV vaccine deferred the tests. In January 1967, Cutter Laboratories, while preparing the E medium U.S.P. lot 1668 from concentrate 35459, manufactured an experimental alum-adsorbed lot CC 52357A, containing 2 x 10^9 formalin-killed virulent P. pestis (total nitrogen 0.334 mg/ml; phenol 0.52%, formalin 0.030% and Al_2O_3 0.069%). In the standard mouse potency test, it yielded a ratio of 10, while that of the aqueous U.S.P. vaccine was 4.0. Both preparations were held for nine months at ±4°C before use in the clinical trials under consideration. On the 28th day after intramuscular inoculation of 1 ml of alum-adsorbed vaccine, only 20% of 24 human subjects showed MPI below 10 and there was none below 5 (Table 4). Used 12 months after storage, it proved inferior to the U.S.P. Cutter vaccine in booster efficacy. The mouse potency ratio determined with a new standard (Ref. 2) did not change; in fact it improved from 1:2.5 to 1:4. Only 17% of the MPI were below 5 in 60% of the reactors with MPI below 10. Previous experiences

HISTOGRAM SHOWING PERCENTAGES OF SIGNIFICANT MPI* IN HUMAN SUBJECTS INOCULATED WITH AQUEOUS, ALUM-COATED SUSPENSIONS OF FORMALIN KILLED VIRULENT P. PESTIS, FRACTION I ANTIGEN AND FREEZE-DRIED ATTENUATED E.V. (S)



(see histogram columns A and B) proved that human subjects inoculated and properly boostered with fresh alum-adsorbed vaccines responded with indices three to four times higher than the indices of those treated with aqueous P. pestis suspensions. Until the factors responsible for the apparent deterioration of the immunogenic potency of alum-adsorbed plague vaccine in storage have been determined and a stable preparation manufactured, the present U.S.P. plague vaccine (E medium) is the only plague prophylactic available for mass inoculations of Armed Forces personnel. Since at best the two-dose inoculation schedule adopted by the military stimulates protective antibodies considered adequate to confer relative immunity against fatal bubonic plague in only 65% of human subjects, the question persists: How can protection indirectly measured by serum antibodies be brought to a level of 90% with inoculations which are safe and free from side effects?

The formalin-killed virulent plague (U.S.P.) Cutter vaccine given in doses exceeding 3 billion plague bacilli, capable of stimulating 60 to 70% MPI below 5, produces unpleasant local reactions of varying intensity and occasionally severe systemic reactions. It was thought that the highly immunogenic, stable, atoxic plague protein Fraction 1 might eliminate the side effects and other problems of active immunization of man against plague. Pilot experiments on small groups of men conducted in 1951-53 (see Reports #2 and #3) indicated that primary intradermal, subcutaneous and intramuscular inoculations of Fraction 1 were well tolerated. Indirect evidence of immunity was reflected in MPI below 10 in as many as 75% and below 5 in 28% of the men inoculated by the intradermal route (see Histogram). Intramuscular inoculations were less antigenic. Re-inoculation with 0.1 to 1 mg of Fraction 1 by either the intradermal or subcutaneous route provoked in some individuals reactions which could have been avoided by intramuscular administration of the antigen. Of all the vaccines and antigens tested, the antibody-priming immunity mechanism of Fraction 1 was of the highest order recorded. Regardless of dosage and mode and schedule of inoculation, the average MPI of human subjects distributed among four entirely independent groups was raised from 40% to 100% below 10 and from 5% to 93% below 5. Such levels had never been achieved with whole vaccines. Experiments to test the correlation of these titers with resistance to plague infection in Macaca mulatta were inconclusive. Protection tests on langurs, and particularly on African green monkeys, with Fraction 1 as the exclusive vaccine, have not been performed. Further evaluation of Fraction 1 as a vaccine against plague in man was discontinued when Burrows (Nature, 173:1246-1247, 1957) found that in mice Fraction 1 afforded no protection against a noncapsulated mutant that produced no Fraction 1. Subsequently, Lawton (see Surgalla, Ann. N.Y. Acad. Sci. 88:1140, 1960) demonstrated that guinea pigs immunized with microgram quantities of Fraction 1 in oil adjuvant and proven solidly immune to capsulated P. pestis, seemed to be more susceptible to the noncapsulated strain. Increased susceptibility was indicated by a rise in mortality and shorter intervals before death. Studies are urgently needed to confirm this effect on subhuman primates; in particular it is imperative that the virulence of the M23 mutant be measured on the two highly susceptible species. Does noncapsulated _P. pestis_ play a role in the epidemiology of plague? This question may be answered to some extent when the plague bacilli isolated in Vietnam from rodent and human subjects are examined.

II. Clinical trials on human subjects and indirect appraisal of the immunogenic efficacy of freeze-dried EV 76 (WR-S)* living attenuated P. pestis vaccine.

Results of the evaluation of the immunogenic efficacy of the freeze-dried live attenuated P. pestis strain EV 76 (WR - Saigon isolate, lot 7) in guinea pigs and subhuman primates were discussed at the meeting of the Commission on Immunization and with Colonel William L. Gochenour, Jr., Deputy Director of the Walter Reed Army Institute of Research, and his associates. It was decided that the Army Research units would evaluate the vaccine by intradermal administration to human volunteers, while the principal investigator (K. F. Meyer) would conduct similar trials on human subjects at the California Medical Facility, Vacaville, with comparable doses of vaccine inoculated subcutaneously. The serologic response to inoculation would be measured by the customary battery of in vivo and in vitro tests. Particular attention would be given to local and systemic reactions in the human subjects immediately after inoculation and for the following 12 days.

(1) Trial with small subcutaneous doses.

On May 8, 1967, the contents of five vials of freeze-dried vaccine reconstituted in saline were pooled and diluted to low-, medium- and high-concentration lots of viable P. pestis EV (WR-S). Three groups of five human subjects were each inoculated with 0.5 ml of a saline vaccine suspension containing 535,000, 5,350,000 or 53,500,000 viable organisms. In Table 5, Parts 1, 2 and 3, clinical reactions and serologic responses are summarized in abbreviated form according to the scheme used for the past 20 years. Only the medium- and large-dose inoculations produced erythema, which persisted for 48-72 hours. A slightly painful but rapidly fading induration developed in one subject (No. 5). The dose of vaccine containing the largest amount (53,500,000) of viable P. pestis stimulated a slight rise in white blood cell counts 24-48 hours after inoculation, and some axillary lymph nodes became palpable. No rise in temperature or general malaise was recorded. Compared with reactions induced by dead plague vaccines and observed during the past 20 years, these local and systemic reactions, with the one exception, were less severe than those induced by typhoid inoculations or large doses of dead plague vaccines.

The antibody response, measured on the 8th, 16th, 29th and 46th days after inoculation, was disappointing, except in one subject who

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*W = Walter, R = Reed (Army Medical Center), S = Saigon, prepared from an isolate in liquid culture obtained from the Pasteur Institute of Saigon in 1965, grown on veal infusion agar at 28°C, suspended in 0.2 molar phosphate containing 7.5% sucrose, 0.7% glutamate and 2.5% human serum albumin.

TABLE 5 - PART 1

Local, Systemic and Serologic Responses of Human Subjects Inoculated Subcutaneously with Small Doses of Freeze-Dried Living Attenuated *P. pestis* EV (WR-S).

First booster inoculation with living EV (WR-S) on 49th day and second booster on 196th day with 1.0 or 0.2 ml dead Army vaccine (Cutter E medium).

Basic immunization: May 8, 1967, EV 76 (WR-S), subcutaneously.

		Local and		Serologic Reactions						
	Viable	Systemic	Normal	8th day		29th day		46th day		
Number	Dosage	Reactions	MPI	HA	CF	HA	MPI	HA	CF	MPI
1. Ki (see Quentin 1951)	535,000	0	8	64	0	64	9	128	0	5
2. Ce	"	0	12	0	0	0	15	0	0	12
3. Vi	"	0	13	0	0	0	17	0	0	17
4. Ga	"	0	15	0	0	0	15	0	0	17
5. Ho	"	15 E	14	0	0	0	17	0	0	16
6. Wa	5,300,000	0	12	0	0	0	12	0	0	12
7. Fl	"	12x20 E	17	0	0	0	17	0	0	21
8. Mit	"	60x60 E	A	0	0	0	A	0	0	A
9. Me	"	45x34 E	A	0	0	0	A	0	0	A
10. Wi	"	22x22 E	16	0	0	0	A	0	0	A
11. Ja	53,000,000	55x80 E	ND	0	0	0	ND	0	0	ND
12. Mi	"	50x40 E	16	0	0	4	16	16	0	18
13. Bl	"	48x48 E	12	0	0	0	13	4	0	18
14. Co	"	30x30 E	14	0	0	0	17	0	0	18
15. Ho	"	59x62 E,I	17	0	0	0	16	0	0	17

* - Dropped from series
A - lab accident; data not available
E - local erythema; extent in millimeters
ND - Not Done
I - Induration
T - Tender on touch
NR - No systemic reaction
WC - White cell count
R - Reaction
MPI - Mouse Protection Index
S - Systemic Reaction

TABLE 5 - PART 2

Local, Systemic and Serologic Responses of Human Subjects Inoculated Subcutaneously with Small Doses of Freeze-Dried Living Attenuated *P. pestis* EV (WR-S)

First Booster: June 26, 1967, EV 76 (WR-S) subcutaneously, 49 days after primary inoculation.

			Serologic Reactions							
			8th day		28th day			217th day		
No.	Booster Viable Dosage	Local and Systemic Reactions	HA	CF	HA	CF	MPI	HA	CF	MPI
1.	4,450,000	NR	64	0	256	0	6	*-	-	-
2.	"	NR	0	0	0	0	15	0	0	18
3.	"	NR	0	0	4	0	15	0	0	18
4.	"	E,NR	0	0	16	0	14	0	0	ns
5.	"	E,NR	0	0	0	0	13	*-	-	-
6.	"	E,NR	0	0	8	0	14	0	0	17
7.	"	E,NR,WC>	0	0	0	0	18	0	0	16
8.	"	E,NR	0	0	0	0	A	4	0	17
9.	"	NR	0	0	0	0	9	0	0	16
10.	"	NR,WC>	0	0	0	0	A	*-	-	-
11.	"	NR	0	0	*-	-	-	-	-	-
12.	"	NR	0	0	16	0	19	0	0	18
13.	"	NR	0	0	16	0	13	0	0	15
14.	"	NR	0	0	0	0	14	0	0	17
15.	"	NR	0	0	8	0	18	0	0	17

ns - no serum

TABLE 5 - PART 3

Local, Systemic and Serologic Responses of Human Subjects Inoculated Subcutaneously with Small Doses of Freeze-Dried Living Attenuated *P. pestis* EV (WR-S)

Second Booster Dose: 1 ml alum-adsorbed Cutter plague vaccine Lot 196. January 8, 1968, 245 days after primary inoculation and 6 months after first booster.

			Serologic Reactions									
			242nd Day	8th day		16th day			28th day			357th day
No.	Second Booster Dose	Local and Systemic Reactions	MPI	HA	CF	HA	CF	MPI	HA	CF	MPI	MPI
2.	1.0 ml	E	19	16	0	64	0	15	16	AC	17	16
3.	"	0, I	14	0	0	128	0	15	64	0	15	20
4.	"	0, I	15	4	0	512	0	9	256	0	11	15
6.	"	0, I	16	512	0	4096	ND	5	256	0	3	6
7.	"	E, S	16	0	0	128	0	14	64	0	14	17
8.	"	E, I	17	16	0	64	0	15	128	0	13	15
9.	"	0, I, T	16	0	0	16	0	14	128	0	10	14
12.	0.2 ml	0, T	21	16	0	64	0	14	128	0	14	16
13.	"	0, T	19	16	0	64	0	17	128	0	16	15
14.	"	E	20	32	0	32	0	22	16	0	17	16
15.	"	0	20	32	0	256	0	16	256	0	12	15

had been vaccinated with dead vaccine in 1951 and in whom hemagglutinins developed. With the highly sensitive titration technique using formalin-treated sheep red blood cells coated with Fraction 1 protein, three men inoculated with the high-concentration doses were found to have low hemagglutinin antibody (HA) titers on the 16th day. The mouse protection index (MPI) antibodies before and after inoculation remained essentially the same.

On the 49th day after basic inoculation, the 15 human subjects were each reinoculated with 0.2 ml of reconstituted attenuated plague vaccine containing 4,450,000 viable P. pestis EV 76 (WR-S).

Four men displayed fleeting erythemas lasting for 24 hours, but no induration, malaise or rise in temperature was observed (see Table 1, Part 2). The white blood cell count rose above 10,000 in two subjects. The small booster inoculation failed to stimulate HA and MPI within 16 days after the inoculation, except in the man given plague vaccine in 1951. By the 28th day, five subjects had low titers (1:8-1:16) of HA antibodies but there were no significant changes in the MPI except in one (No. 9).

Two hundred and forty-five days after the basic dose, or 196 days after the first booster inoculation, the 11 subjects remaining in the three groups vaccinated with EV 76 (WR-S) were reinoculated with alum-adsorbed Army vaccine (either 1 ml 2 billion, or 0.2 ml 400,000,000 formalin-killed virulent P. pestis 195/P). The inoculations were well tolerated (Table 5, Part 3). Aside from slight erythemas in four subjects, local reactions were characterized by moderate indurations and tenderness to touch. One man (No. 8) complained of malaise but had no rise in temperature.

Any dead vaccine containing Fraction 1 inoculated intramuscularly in a 1-ml amount generally causes a definite though variable, and in some individuals vigorous, HA antibody response. The high titer in human subject No. 6 was accompanied by a low MPI of 5 by the 16th day and 3 by the 28th day. It persisted for four months--the longest observation period. A similar correlation was noted in the serum of subject No. 4, who had an HA titer of 1:512 accompanied by an MPI of 9. Observations from several clinical trials with the alum-adsorbed dead plague vaccine reveal that following a single basic inoculation of 1 ml, passive mouse protection titers with few exceptions are lowered and 9, or 30%, are 10 or below and either none, or at best 5%, is below 5. In response to a booster inoculation, the percentage of MPI below 10 promptly rises to 60-65% and that below 5 to 18-25%. By contrast, 11 human subjects in Phase 3 of their course of plague inoculations responded serologically in the same manner as those never in contact with plague antigens.

In summary, this clinical trial demonstrated thatthe sample of freeze-dried living attenuated P. pestis EV 76 (WR-S) vaccine in the highest-concentration dose of 53 million viable organisms inoculated subcutaneously fails to stimulate mouse protective antibodies. It does not sensitize. No booster dose effects were elicited with either the live or the dead alum-adsorbed plague vaccine of low immunogenicity.

(2) Clinical trial with standard large doses.

Introduction: The disappointing immunogenic response of human subjects to low-dose subcutaneous or intracutaneous inoculations (trial by the Walter Reed Institute of Research) is reminiscent of previously unpublished reports. Girard (Arch. Institut Pasteur, Madagascar, 1935-36) reports that he inoculated each subject subcutaneously with 1 ml of his EV 76 vaccine containing 1,000 million viable attenuated P. pestis in a total of 2 billion. The Soviet immunologist, E. I. Korobkova (Live anti-plague vaccine, Moscow, 1956, and Zh. Microbiol. 33:76-82, 1962) states that an effective dose of EV vaccine, if administered subcutaneously to an adult male, must contain 1.5 billion P. pestis determined by optical methods and must on culture yield no less than 450,000,000 viable organisms in the inoculated vaccine dose. No carefully controlled trials followed by serologic tests on human subjects have been reported using such vaccine doses except for one test reported to the Commission on Immunization (Annual Report #3, 1952-53, Table 9, p. 36, repeated for convenient reference).

The customary dose of 1,000 million containing 340 million viable attenuated P. pestis of the original Girard EV 76 obtained from Madagascar in 1951, given subcutaneously between the scapulae, caused the following reactions: "All of the group, varying in age from 21 to 59 (average 33 years) experienced marked anorexia. The temperature varied from 98.6° to 103.6°F at the end of 24 hours. The one man who had a normal temperature at the end of 24 hours had a temperature of 101.6°F at the end of 72 hours. Half of the group had normal temperatures at the end of 72 hours and two had a temperature of 100°F on the 120th hour. All temperatures were normal at the end of the 144th hour. All appeared ill and requested cell passes so that they could rest in bed. Two, on advice of Dr. M. D. Willcutts, were hospitalized for 48 hours because of systemic reactions. The reactions at the point of injection were confined to hot, erythematous, moderately indurated, painful regions, with axes measuring 5 and 6 cm. In one volunteer a carbuncle-like lesion developed between the scapulae but disappeared within 6 days." Lieutenant James E. Whiteside, M.D., U.S.N., who assisted in these studies at San Quentin, appraised the results as follows: "I feel that vaccination with the avirulent strain EV (transplant obtained in 1951) is impractical because almost all the men inoculated were unable to work for at least 24 hours, and half of the group were at least partially incapacitated for as long as

72 hours. For mass vaccination of military personnel this would be impractical."

For the purpose of measuring the immunogenic efficacy of a large dose of freeze-dried EV (WR-S) plague vaccine, it was decided to inoculate 25 human subjects with a reconstituted pool of vaccine prepared from eight vials (Lot 7, four originally obtained in 1966 and four in 1967). The final inoculum dose of 0.5 ml contained 420,000,000 viable *P. pestis* EV (WR-S) in a microscopic-chamber-counted suspension of a total 2,050 million bacterial cells. None of the human subjects had antibodies for *P. pestis* Fraction 1 or prior to the trial gave a history of previous plague vaccination. During the six days after vaccination they were observed at three-hour intervals, blood samples for white cell count were taken daily, and temperatures recorded twice a day. Local and systemic reactions are detailed in Table 6, Part 1. Within a few hours after careful inoculation of 0.5 ml of the vaccine suspension into the subcutaneous tissue of the deltoid region, all subjects developed erythematous areas of varying extent and intensity. In general the areas were from 4 to 10 cm wide and became indurated, hot and painful, or at least tender to touch. One subject (No. 10) developed a red, painful, edematous area extending over the elbow and impairing the movement of the lower arm. Though the erythema receded in most within 72-96 hours, by the 10th day after inoculation a hard, sometimes tender area or a small, indurated nodule was readily palpable below the needle puncture. In one subject the superficial skin layers over the nodule desquamated. Usually edema, when present, persisted for up to 96 hours. By the 24th hour the axillary lymph nodes of each individual were definitely palpable; some were enlarged and slightly tender. Except in the case of two subjects, local inflammatory reactions and general discomfort were apparently borne cheerfully by the vaccinated men, since they continued their usual daily activities. Subject No. 10, who had impaired function of his arm and general malaise, gladly accepted the advice to rest for 24 hours. Subject No. 1 developed a fluctuating indurated area covered by desquamated layers of skin at the inoculation site; muscle function was slightly impaired until the small abscess spontaneously ruptured on the 19th day. A swab smear of the exudate collected in the infirmary yielded coagulase-positive staphylococci. The lesion healed rapidly and the induration promptly disappeared. Six men had definite rises in temperature during the first two days, while the white blood cell count rose in eight. Malaise, headaches and myalgia were experienced during the first 48 hours by approximately 50% of those vaccinated. Grading the reactions according to the standard procedure in use for the past 20 years permits comparisons (Table 6, Part 3). The freeze-dried attenuated living *P. pestis* EV (WR-S) incapacitated 1 of 25 human subjects for only 24 hours. By contrast, in 1951 the Girard EV 76 inoculated in a similar dose incapacitated six individuals with fever and general malaise, and eight with myalgia and headache for 48 hours. Two had to be hospitalized for two days. Local reactions were not marked, since the inoculations had been made between the shoulder blades.

TABLE 6 - PART 1

Clinical and Serologic Reactions Observed in a Group of 25 Human Subjects Inoculated Subcutaneously with 420,000,000 Viable* Freeze-Dried Living EV 76 (WR-S)

*Total count - 2 billion

Basic immunization: July 10, 1967

	Clinical Response			Serologic Reactions							
	Local			Normal	8th day		16th day		28th day		
Number	Reaction	Fever	WBC	MPI	HA	CF	HA	CF	HA	CF	MPI
2. Le	+++ L	+		23	64	0	256	0	128	0	13
3. Sn	+ L	+	+	20	4	0	128	0	32	0	17
4. La	++ L			(8)	4	0	32	0	0	0	(11)
5. Tu	++ L	+		20	8	0	64	0	0	0	10
7. Ru	+ L		+	16	16	0	512	0	128	0	15
8. Po	+ L			13	0	0	64	0	32	0	12
9. La	+++ L			19	4	0	256	0	512	0	16
10. Ru	+ L	+	+	20	32	0	256	0	256	0	16
11. Wi	+ L			14	32	0	64	0	256	0	13
12. Re	+ L		+ 10 day	12	4	0	32	0	32	0	15
13. Ha	+ L		+	17	4	0	4	0	128	0	16
14. Ma	+ L			14	0	0	8	0	0	0	13
15. Ne	+ L			17	4	0	0	0	0	0	15
17. Sm	++ L	+		19	16	0	128	0	128	0	16
18. Re	+ L			17	32	0	256	0	256	0	13
19. Pa	+ L			14	0	0	0	0	0	0	16
21. Wh	+ L		+	14	4	0	8	0	0	0	9
22. Ha	+ L		+	20	16	0	128	0	256	0	15
24. Em	+ L			19	8	0	64	0	128	0	16
25. Ro	+ L			15	0	0	32	0	16	0	16
27. Ni	+ L			21	4	0	32	0	32	0	21
28. Mo	++ L	+		13	4	0	256	0	256	0	14
30. St	+ L			18	0	0	64	0	128	0	18
31. Ma	+ L			15	8	0	128	0	64	0	15
32. Hu	+-L			14	4	0	32	0	16	0	17
Average:				16.5			104		111		14.3

() - mix-up in cages
L - lymphadenopathy
MPI - Mouse Protection Index
HA - Hemagglutination antibody
CF - Complement fixation

TABLE 6 - PART 2

	Serologic Reactions									
	45th day			60th day			84th day		115th day	
No.	HA	CF	MPI	HA	CF	MPI	HA	CF	HA	CF
2.	16	0	15	8	0	14	8	0	8	0
3.	32	0	16	0	0	13	8	0	16	0
4.	8	0	(11)	0	0	(16)	0	0	0	0
5.	32	0	15	0	0	16	16	0	0	0
7.	128	0	22	64	0	20	256	0	128	0
8.	16	0	10	4	0	12	16	0	8	0
9.	128	0	12	32	0	14	256	0	64	0
10.	64	0	14	32	0	14	128	0	32	0
11.	32	0	16	0	0	14	64	0	0	0
12.	16	0	13	4	0	14	8	0	4	0
13.	64	0	17	0	0	17	8	0	*-	-
14.	0	0	9	4	0	14	0	0	0	0
15.	0	0	19	0	0	14	0	0	0	0
17.	8	0	16	0	0	17	0	0	0	0
18.	32	0	22	0	0	16	0	0	0	0
19.	0	0	12	0	0	13	0	0	0	0
21.	16	0	15	0	0	8	4	0	4	0
22.	16	0	14	0	0	18	8	0	0	0
24.	32	0	17	4	0	19	4	0	0	0
25.	16	0	22	4	0	21	8	0	4	0
27.	0	0	21	0	0	21	0	0	0	0
28.	64	0	14	16	0	12	16	0	4	0
30.	32	0	18	8	0	19	16	0	0	0
31.	16	0	10	4	0	16	4	0	0	0
32.	4	0	18	0	0	16	8	0	8	0
Average:			15.5							

*Dropped from series

TABLE 6 - PART 3

Grading of Clinical Reactions of 25 Human Subjects Inoculated Subcutaneously and Intramuscularly With Living Attenuated and Killed Plague Vaccines

	24 hours						48 hours						72-96 hours					
	Local			General			Local			General			Local			General		
	N	M	S	N	M	S	N	M	S	N	M	S	N	M	S	N	M	S
Freeze-Dried EV 76 (WR-S) 420 million living, subcutaneous, 25	0	24	1	17	7	1	0	24	1	16	6	3	3	22	0	19	6	0
Killed Plague Vaccine E Aqueous, 1 ml intramuscular, 25	21	4	0	22	3	0		-			-		23	2	0	25	0	0
Killed Plague Vaccine alum-adsorbed 1 ml intramuscular, 25	20	5	0	21	4	0		-			-		23	2	0	25	0	0
Killed Plague Vaccine alum-adsorbed, 1 ml, 21		-			-		14	6	1	17	3	1		-			-	

Local - None: No erythema, induration or edema; slight tenderness to touch disappearing within 24 to 48 hours.

Mild: Erythema (2 x 4 cm) but little or no induration; tenderness persisting longer than 24 hours, sometimes with erythema.

Severe: Marked local edema of entire arm, extending to forearm; moderate or marked erythema, with or without lymphadenitis and lymphangitis.

General - None: No temperature elevation, chilly sensations, headache or general symptoms.

Mild: Headache, chilliness, myaglia, general malaise or temperature elevation of more than 1°F not explained by a respiratory infection or other cause.

Moderately Severe: Chills and headache; temperature about 100.6°F; general incapacitation.

TABLE 6 - PART 4

Clinical and Serologic Reactions Observed in 28 Human Subjects Inoculated July 10, 1967 With 420 million Viable Freeze-Dried EV (WR-S)

Booster inoculation: 181st day, 6 months - January 8, 1968

Group I: EV (WR-S) 8.45 million viable, 27.66 million total in 0.5 ml subcutaneously
Group II: Army vaccine 0.2 ml intramuscularly
Group III: Army vaccine 0.2 ml intradermally
Group IV: Fraction 1, 100 micrograms in 0.2 ml, intradermally

Group and Number	Local Reactions	Fever	Average WBC	L.N.
I. 8	34 x 40 E, I, T	-	6100-10,000	++
11	25 x 30 E, I, T	-	6100-9600	++
12	18 x 27 E, I 24, T	-	7500-10,100	-
14	30 x 27 E, I, T	-	7400-9600	-
15	30 x 20 E, Nod 15, 34 I	-	6100-10,600	-
17	28 x 26 E, Nod 37, T	-	8800-10,600	+
18	18 x 14 E, Nod 20	-	9300-9700	-
19	18 x 25 E, Nod 20	-	6900-9800	+
21	20 x 20 E, I, 51 Nod	-	6600-12,400	+
22	0 0	-	7500-10,900	-
II. 9	0 0			
10	65 x 40 E, I			
25	0 I			
28	0 I 30, (12) I			
III. 27	8x10 E, I(center). (12) 4x4 N			
30	16x19 E, I(center). (12) 14x10 E,N,I			
31	18x20 E, I(center raised). (12) 9x8 E,N			
32	38x35 E, I, N. (12) 14x16 N, eschar			
64 C	34x31 E, I,N 18. (12) 13x16 E,I,N			
65 C	20x31 E, I,N 18. (12) 18x12 I, N raised			
IV. 2	25 x 25 0			
3	10 x 12 0 E			
4	8 x 8 0 E			
5	29 x 22 0 E			
7	9 x 9 0 E			
61 C	8 x 8 0 E. (12) 0			
62 C	8 x 10 0 E. (12) 0			
63 C	0 0 (12) 0 0			

C - Control
E - Erythema
I - Induration
Nod, N - Nodule
T - Tender
(12) - 12th day
-, 0 - None
L - Lymphadenopathy

TABLE 6 - PART 5

Group and Number	Serologic Reactions Pre-booster 179th day HA	 CF	 MPI	 Post-Booster 8th day HA	 CF	 16th day HA	 CF	 28th day HA	 CF	 MPI	 56th day HA	 CF	 80th day HA	 CF
I. 8	4	0	15	0	0	0	0	8	0	16	0	0	4	0
11	0	0	12	4	0	0	0	16	0	12	0	0	4	0
12	0	0	16	0	0	0	0	0	0	16	0	0	4	0
14	0	0	16	0	0	0	0	0	0	15	0	0	0	0
15	0	0	16	0	0	0	0	0	0	15	0	0	128	0
17	0	0	18	0	0	0	0	0	0	19	0	0	0	0
18	0	0	13	4	0	4	0	8	0	14	0	0	8	0
19	0	0	16	0	0	0	0	0	0	19	0	0	0	0
21	8	0	15	4	0	4	0	16	0	12	-	-	-	-
22	4	0	11	0	0	0	0	8	0	14	0	0	4	0
II. 9	4	0	15	8	0	512	0	512	0	1	32	0	128	0
10	4	0	14	16	0	0	0	256	0	10	32	0	-	-
25	4	0	13	8	0	256	0	128	0	14	-	-	-	-
28	4	0	12	64	0	32	0	128	0	8	0	0	64	0
III. 27	4	0	14	8	0	16	0	128	0	18	0	0	4	0
30	0	0	15	32	0	256	0	256	0	17	16	0	64	0
31	0	0	19	32	0	128	0	256	0	19	16	0	64	0
32	0	0	18	8	0	16	0	8	0	17	0	0	4	0
64 C	0	0	15	16	0	64	0	64	0	16	4096	0	4	0
65 C	0	0	13	4	0	64	0	128	0	17	32	0	0	0
IV. 2	4	0	15	512	0	4096	0	8192	0	10	32	0	1084	0
3	0	0	15	8	0	128	0	128	0	16	0	0	64	0
4	0	0	14	16	0	256	0	256	0	7	16	0	256	0
5	4	0	15	64	0	2048	0	4096	0	6	1024	0	2048	0
7	0	0	17	0	0	64	NT	128	0	14	0	0	16	0
61 C	0	0	18	64	0	512	0	512	0	6	1024	0	256	0
62 C	0	0	19	64	0	256	0	512	0	12	64	0	4	0
63 C	0	0	18	32	0	64	0	64	0	11	0	0	8	0

Pre-booster	MPI -		
		Group I:	17
		Group II:	16
		Group III:	16
		Group IV:	14

Until 1945 when Rotman (J. Roy. Nav. Med. Serv. 31:155-158, 1945) called attention to his observations indicating that the EV 76 vaccine used during an outbreak of plague in Dakar, French Congo, produced severe local and systemic reactions, these side effects were never mentioned in previous reports. For quick reference, pertinent paragraphs are herewith quoted verbatim. "An intensive vaccination campaign was embarked upon by the authorities using a living vaccine, the attenuated strain of *P. pestis* used in the Madagascar outbreak of 1938 and manufactured at the Institut Pasteur, Dakar. Over 180,000 natives and 20,000 Europeans were vaccinated and although many Europeans' reactions were severe, both systemic and local, no mortality was recorded. A 1 ml was given in the scapular region and many people had tissue sloughs at the site of inoculation. Each individual vaccinated was provided with a *passport sanitaire* which allowed free movement in and out of town and later on the issue of a new food ration card."

"The mortality rate amongst the natives was 85 per cent, and was mostly confined to the lower types who were living under very squalid and unsanitary conditions. Amongst *those vaccinated over a period of fifteen days which numbered 5 per cent of total cases the mortality rate was 66 per cent*. By the end of July, a total of 135 cases with a total mortality of 116 were reported with three European cases, all fatal; the cases occurring at the rate of four to five a day."

"Foreign personnel, i.e., British and American, received inoculations of killed vaccine (Lister Institute), which had a modifying effect upon the French vaccination received later. The French medical authorities had no faith in the killed vaccine, and all observers who were in and out of the infected areas had to be vaccinated with the living vaccine."

In her monograph on live antiplague vaccine (1956), Korobkova states (translated) "Live plague vaccine produces a local and a general reaction in inoculated persons, the severity of which is dependent on the characteristics of the body, its reactivity and the method of vaccination. In general, it should be recognized that the severest reactions which accompany inoculations of live vaccine are considerably weaker than the reactions to killed Haffkine vaccine or to typhoid-paratyphoid vaccine." To support this statement, she quotes S. I. Zlatogorow (1922) as follows: "The injection of Haffkine vaccine subcutaneously produces pain at the site of injection after four to eight hours, which reaches its greater intensity after 24 to 48 hours. At the same time, a tumor and cutaneous erythema develop at the injection site. The regional lymphatic glands not uncommonly swell up, the temperature rises to 38.5°-39.5° and remains this way for 24 to 48 hours. General weakness, headache, and loss of appetite occur over the course of the first day and can be so severe that the person has to lie in bed. Those inoculated sometimes give the impression of being seriously ill. The tumor at the injection site often remains for several days and comes down to normal only gradually. Less frequently, those inoculated show an insignificant general and local reaction."

From her own experience, Korobkova lists the following observations: "In response to subcutaneous inoculations of EV vaccine, as a rule, local reactions of different degrees of intensity occur in almost 100 per cent of cases. Depending on the individual characteristics of the body, at the injection site of the vaccine an inflammatory reaction is observed in the form of swelling, marked redness, the area of which sometimes reaches the dimensions of 15 x 15 centimeters, and individual cases even larger, moderate painfulness, chiefly tenderness on palpation; the skin becomes stretched and hot to the touch. The regional lymphatic glands not uncommonly enlarge, although in the majority of cases they cannot be felt. A febrile condition accompanied by a rise in temperature above 38^{0}, weakness, headaches are noted in a small percentage of those inoculated. In less frequent cases, the reaction is accompanied by gastro-intestinal tract signs, diarrhea, nausea. Reactions of this type are seen more frequently in persons who usually suffer from intestinal disorders, and they are brief.

"Reactive phenomena of local character after the subcutaneous injection of the vaccine begin to manifest themselves as early as four to five hours after the inoculation and attain their complete development at 24 to 48 hours; in less common cases the reaction develops at 30 to 40 hours. After 48 hours, the regression of the inflammatory signs begins: the erythema even though it becomes more diffuse, fades, the painfulness and tenderness subside, the swelling disappears, and after three to four days a small thickening remains at the site of the injection which is resorbed after eight to ten days. The elevated temperature, if it occurs, remains for several hours (10 to 15 hours); the other reactive phenomena of general character disappear even more quickly. Severe reactions, where the one inoculated has to lie in bed, occur very rarely."

In a section entitled "Post vaccinal reactions", R. Pollitzer (Plague and Plague Control in the Soviet Union, History and Bibliography through 1964, The Institute of Contemporary Russian Studies, Fordham University, New York 1966, p. 247) reviews the Soviet observations of the reactions caused by administration of live plague vaccines. He makes no critical analysis of the numerous articles condensed and reviewed. After 1954 the intracutaneous and subcutaneous administration of EV vaccine was replaced by cutaneous and by aerosol vaccination. The conclusions of several authors deserve brief consideration. Ziusin (Reference 713, 1959) concludes: "Intracutaneous and subcutaneous anti-plague vaccination with vaccine strains 1-17 leads to considerable general and local reactions with a loss of the working capability for 1-3 days. In 2 out of 230 instances of intracutaneous vaccination the formation of sterile abscess resulted." Cutaneous vaccination produced the slightest general and local reactions and therefore this mode of immunization is to be recommended as the main method for conducting mass vaccinations. The EV vaccine was less productive of reactions when administered subcutaneously. To compare reactions caused by aerosol immunization against plague and those resulting from EV vaccine administration by the usual routes, Aleksandrov and his associates (References 16, 17 and 18, 1962-1963) vaccinated 100 healthy 18-25-year-old men subcutaneously. They noted that among the 100, a general post-vaccination reaction was invariably present, leading to fever above 38.5°C in 66, temperature rises ranging from 37.6 to 38.5°C in 26, and slight elevations of the body temperature (37.7-37.5°C) in 8 of the vaccinated. Local reactions (hyperaemia, swellings, and tenderness at the site of vaccination and sometimes involvement of the regional lymph nodes) were observed in 98 of the vaccinated, lasting for two to seven days. After a single administration of one human dose (2 billion organisms for the given vaccine lots), general post-vaccination reactions were recorded, depending upon the various vaccine lots; in lot 41-100%, lot 16-92% were moderately severe or severe in character. Repeated subcutaneous vaccination with a lesser dose (1.2 billion organisms) produced less frequent general reactions in 28.1%, but local reactions were evident in 96%. A statement by Shmuter et al. (Ref. 581, 1959) referring to cutaneous vaccination propounds the following concept: In the vaccinated, the character of the reactions, their intensity, and especially their duration, are to a considerable degree related to the percentage of live cells in the vaccine. The administration of a vaccine with a high percentage of viable cells produces more marked reactions. Finally, Koslov and his collaborators (Reference 293, 1960) record useful information on the reactions of individuals to cutaneous injection of dried live vaccine. Most of the 136 women and 155 men each vaccinated with 1.5 x 10^9 organisms exhibited general malaise and headache lasting two days; 18% manifested pyrexia up to 39.4°C for 18-20 hours. Regional lymph nodes were enlarged and tender in 8%. More severe reactions occurred in only 2%. Local reactions at the site of injection varied in different individuals, but in most cases hyperaemia of the skin and an area of infiltration (25-100mm) developed, lasting two to three days. A papule progressing to a pustule appeared six to eight hours after injection and disappeared in five to seven days.

Girard (L'immunité dans l'infection pesteuse, La Biologie Médicale, 52:75, 1963, "Epilogue") quotes the translated conclusions recorded by Dr. J. Smadel at the time of their visit to Madagascar in 1951 as follows:

"According to the description of the French workers and our own observations, the local and general reactions in persons who have received an injection of EV vaccine are a little more severe than those brought on by the classical antityphoid vaccine. They are more pronounced in Europeans, after subcutaneous injection in the latero-posterior surface of the forearm than after subcutaneous injection above the shoulder blade. The first route is used in the Malagasy* to avoid having to ask them to disrobe, and the second in Europeans. Nevertheless, the Malagasy are less sensitive because, after injection into the forearm, they have a less intense reaction than the Europeans inoculated in the shoulder. Intramuscular injection is prohibited because of the severity of the local reactions. On the other hand, the tests carried out in order to obtain a proof of the proliferation of the EV germs in human subjects gave negative results, despite the fact that under several conditions we found, after the inoculations, a few live germs at the injection site or at the level of the regional ganglionic chain (some data on this point may be found in the Archives de l'Institut Pasteur of Tananarive, years 1937 and 1938)."

A comparison of reactions induced by live and dead vaccines in human subjects can be made only with reservations, since the dead vaccines were inoculated intramuscularly (see Table 6, Part 4). The absence of systemic reactions, particularly fever, in those inoculated with formalin-treated antigen is noteworthy. Local reactions in the form of painful indurations rarely exceed 20% or persist longer than 96 hours in groups inoculated with Army vaccines, while reactions of varying intensity and prolonged duration are characteristic for live vaccine. By Armed Forces standards, freeze-dried living attenuated P. pestis EV 76 (WR-S) administered subcutaneously in the recommended 500 million dose is unsuitable in its present form for mass inoculation of military personnel.

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*"The choice of the forearm for a subcutaneous vaccination may appear abnormal, but it was adopted for several reasons: ease of execution during the gatherings of thousands of persons of the two sexes and of all ages who did not have to disrobe; convenient control of the local reactions and of any epitrochlear and axillary ganglionic repercussions which may have developed. However, the Malagasy themselves, many of whom sleep on a mat placed on the ground or on a rudimentary stretcher, complained of pains caused by the pressure of the ground or of the wood when they were vaccinated in the shoulder blade.

"There is no doubt that the local reaction, which is more marked in the forearm than in the dorsal region, is connected with a slower absorption of the vaccine and with a longer persistence of the live germs in the cellular tissue, a fact which, in the final analysis, ought to be beneficial for the immunization."

Antibody responses in the sera of inoculated subjects as measured by three tests, two in vitro and one in vivo, executed on the 8th, 16th, 28th, 45th and 60th days after inoculation are summarized in Table 6, Parts 1 and 2. The average passive hemagglutination titers for Fraction 1 antigen, which appeared by the 16th and 28th day after inoculation of the vaccine in 23/25 and 19/25 human subjects, are of the same magnitude, 104 and 111, as those recorded for the sera of men inoculated with 2.5 billion dead plague bacilli (Annual Report #12, 1962, p. 18). The absence of complement-fixing antibodies is not surprising. The average mouse protection index of the sera collected before inoculation - 16.6 - was reduced to 14 on the 28th day and to 15 on the 45th day after inoculation. Only two sera yielded titers of 10 and below. The trial partly confirms reports from Madagascar (G. Girard, L'immunité dans l'infection pesteuse--La Biologie Médicale 52:42-45, 1963) and the Soviet Union Alma-Ata Stavropol Reports (Oganian, E.F. and Cherchenko, I.I., p. 250, 1966) that the EV 76 vaccine fails to stimulate significant antibody levels. The actual dose of EV vaccine administered to 77 persons in the Alma-Ata series is unknown, since it was applied by the cutaneous scarification method. As these investigators used the rather insensitive filter paper disc method recommended by Trainer et al. (Zoonoses Research 2:2, 1963), it is suspected that low antibody levels could not have been detected.

The only inoculation trial on human subjects with the Madagascar EV 1951 isolate followed by serologic tests was reported in 1952 (Annual Report #2, 9, Table V). At that time it was noted that the vaccine, grown at 37°C, induced unpleasant side effects when given in a dose of 1 billion organisms containing approximately 340 million viable attenuated P. pestis. Nearly all the men inoculated were unable to work for at least 24 hours, and half the group was at least partially incapacitated for as long as 72 hours. Equally disconcerting was the fact that an antibody response was infrequent despite the reactions to inoculation. Twenty-one days after inoculation the average MPI of 12.3 with low complement-fixing antibodies (only one with 1:2) and low agglutinins (not HA) averaging 1:13, was below the range characteristic for human subjects inoculated with one dose of a formalin-killed suspension of virulent P. pestis. Since the men refused to be reinoculated with EV 76, they received single booster inoculations with 1 mg Fraction 1 in 1 ml of saline six months after basic immunization with the live attenuated vaccine. Within 21 days the antibodies rose strikingly. In fact, in four of the nine men (4/9) still available for the test, the MPI was below 2, and the serum from one protected all inoculated mice. The average MPI was 8.6; in addition, CF antibodies in titers up to 1:32 and agglutinins up to 1:64 were recorded. Similar observations have been reported from Madagascar (Payne et al., J. Immunol. 77:24, 1956). The antibody, and probably the immunity-priming mechanism, of the 28°C agar-grown and saline-suspended attenuated EV 76 Madagascar isolate, were apparent.

The booster inoculum of Fraction 1 corresponds to a 1-ml dose of vaccine containing approximately 300 million viable P. pestis.

To verify reactions observed in the course of the 1951 pilot experiment, the 23 human subjects still available from the 1967 studies were divided into four groups and reinoculated on the 181st day after basic inoculation with 420 million freeze-dried viable EV (WR-S) as follows:

Group I

Ten subjects (Nos. 8 to 22 listed in Table 6, Parts 3 and 4) were each inoculated subcutaneously with 0.5 ml of reconstituted EV (WR-S) containing 8.45 million viable and 27.66 million total P. pestis. The dose was twice that used in trial I, but had only one-tenth the number of formalin-killed plague bacilli containing Fraction 1 protein routinely used for booster inoculations in mass immunizations. This particular dose was chosen in order to avoid anticipated serious unpleasant side effects. The inoculated men reacted as follows:

After four hours none had local erythema; by the 24th hour nine had distinct erythematous areas 18 - 40 mm in diameter, the centers indurated and tender to touch. By the 72nd hour the erythemas had faded, but in the center area in each case a slightly tender 20 - 37 mm nodule persisted. One subject complained of headache, but no myalgia or fever was recorded. Axillary lymph nodes were palpable in five subjects. White blood cell counts two to three days preceding the inoculation varied from 5,800 to 9,200 (average: 7,400); all had definite rises--in fact the count was above 10,000 to 12,400 in six (average: 10,330), but returned to normal range within 48 hours (Table 6, Parts 4 and 5). None of the subjects complained of local or systemic discomfort. The rise in white cell count may be interpreted as a modest sensitization reaction.

The immunogenic response to booster inoculation was disappointing. On the 28th day, five of the ten human subjects had HA titers of 1:8 to 1:16 and there was a slight lowering of the MPI in two. Why this failure? (a) The vaccine dose (8 million viable in 27 million total P. pestis), close to the threshold of unpleasant side effects, is incapable of stimulating a booster response; (b) the EV 76 (WR-S) viable vaccine even in a standard dose fails to sensitize, as already observed in the first clinical trial; and (c) the freeze-dried preparation has lost its immunogenic capacity. The latter explanation is partially supported by the observation that the minimum protective vaccine dose required to immunize guinea pigs has progressively increased from 400 viable P. pestis in April 1966 to 70,000 in July 1967 and 84,500 in January 1968.

Group II

Four human subjects (Nos. 9, 10, 25 and 28 in Table 6, Parts 4 and 5) were each inoculated intramuscularly with 0.2 ml U.S.P. plague vaccine grown on E media, or a total of 400,000,000 formalin-killed virulent P. pestis 195/P, the booster dose customarily used in man for prophylaxis against plague. The inoculation produced a 65 x 40 mm diffuse, slightly indurated erythema in one subject by the 72nd hour, but none in the other three. The antibody response expressed in HA conforms to that of human subjects inoculated for the first time with U.S.P. plague vaccine. The low MPI (1.0) correlated well with the moderately high HA (1:512) titer. It may be mere coincidence that three MPI were below 10 and one was even below 5--conclusions cannot be drawn.

Group III

Clinicians seeking to reduce the local reactions caused by subcutaneous injections of plague vaccines in civilians suggested intradermal administration of the booster dose. No controlled observations have been recorded with respect to local and systemic reactions with subsequent serologic examination of the sera of persons thus inoculated. Four human subjects previously vaccinated with EV (WR-S) each received 0.2 ml of the U.S.P. vaccine, very carefully inoculated by the intradermal route with a 25-gauge needle. Within four hours erythematous areas measuring an average of 10-14mm, without edema, surrounded the needle punctures. These reactions increased slightly within 24 hours; in one man the central red nodule was surrounded by a faintly erythematous zone. By the 72nd hour the central area--site of the original bleb--was invariably indurated, raised and tender to touch. Nodules varying in size and slightly purplish in color persisted for two weeks. A small eschar over the nodule had even developed by the 12th day in one instance.

Two subjects with no history of exposure to plague or vaccines had similar local reactions at the site of the bleb within 24 hours. Within 72 hours the nodules were reduced to 12 x 12 mm, slightly indurated areas tender to touch. No differences in diameter of the nodules of vaccinated and control subjects were recorded, but the erythema was less marked in the controls. Antibody response to intradermal inoculation of a small dose of U.S.P. plague vaccine was of the same magnitude in unvaccinated and vaccinated human subjects given a large subcutaneous dose of EV 76 (WR-S) six months previously.

Intradermal inoculation of the vaccine was chosen in order to obtain some evidence that the immune response to plague in man is reflected by hypersensitivity. Koslov et al. (J. Microbiol. Epidem. Immunobiol. 31:1508, 1960) and Korobkova et al. (J. Microbiol. Epidem. Immunobiol. 32:1608, 1961) assessed the immune response to dried live vaccine by the allergic reaction to the microbial pestin, which they claim persists for six months but not for 12 months after vaccination. In preliminary tests a so-called polypeptide-polysaccharide complex P.P., prepared according to the sketchy method described by Korobkova et al., caused uninterpretable skin reactions in subhuman primates and guinea pigs inoculated with EV 76 vaccine. The whole vaccine used as a substitute for the pestin resulted in immediate erythema but no delayed tuberculin-type reactions in vaccinated subjects and comparable but less marked effects in the nonvaccinated. Further studies are necessary to interpret the nature of the reactions. At present it is not established that the immune response in human subjects as evidenced by some type of hypersensitivity can be correlated with immunity to plague.

Intradermal inoculation of dead vaccine induces persisting indurated nodules even with the most careful intracutaneous injection technic. Intramuscular inoculation induces unpleasant local reactions less frequently than the subcutaneous or intradermal administration of U.S.P. plague vaccines. It is recommended that the reaction pattern and antibody response to killed vaccine administered by the jet inoculation procedure as now practiced at the Lackland Air Base be determined as soon as possible.

Group IV

The 1951 trial with the EV 76 Girard attenuated living P. pestis vaccine demonstrated that a booster inoculation of the highly immunogenic plague antigen-Fraction 1 protein stimulated excellent antibody response without local or systemic reactions. Studies reported in 1953 (Annual Report #3) demonstrated that 100 µg of Fraction 1 intracutaneously inoculated reduced the average MPI from 11.8 to 7.1 with only a moderate immediate allergic reaction in approximately 50% of the persons tested. Confirmation of these observations appeared desirable.

Five human subjects (Nos. 2, 3, 4, 5, 7, see Table 6, Parts 4 and 5) who had local and systemic reactions to the large-dose EV (WR-S) vaccine, and three control subjects not known to have previously been vaccinated or exposed to plague, were each carefully inoculated intradermally with 0.2 ml of safety-tested saline solution containing 100 µg highly purified Fraction 1. Within 4 hours these inoculations produced areas of erythema up to 29 x 22 mm with slight edema. In two subjects the merthiolated saline also induced a fleeting erythema which increased slightly and persisted for 24 hours. The reactions had completely faded by the 72nd hour. In subject No. 2, who had developed a local abscess at the site of EV (WR-S) vaccine inoculation, an erythema only faintly visible by the fourth hour increased to a 25 x 25 mm area by the 24th hour. At 72 hours the erythematous area was still visible on his dark skin, but the center was reduced to a small pinhead-sized nodule. Two control subjects injected with the solvent and Fraction 1 solution had fleeting erythemas by the fourth hour, but only one had a faint reaction by the 24th hour. The tests on this small number of vaccinated men furnish no valid evidence that the state of immunity in human subjects vaccinated with large doses of EV (WR-S) can be measured reliably or that the immediate allergic skin reactions may serve as dependable signs of immunity.

The 100-ug Fraction 1 booster did not merely restore the HA antibodies, but in four men (2, 3, 4, and 5) it provoked higher levels (1:2048, 1:4096, to 1:8192) than those recorded from the 8th to the 28th day after subcutaneous inoculation of the large EV (WR-S) vaccine dose. Corresponding with in vitro antibody titers, MPI were below 10 in four of the five men. In fact, the serum of human subject No. 2, who reacted strongly to the vaccine injection, protected all the mice used in the test. Apparently he was sensitized and therefore reacted vigorously. However, the same interpretation cannot be applied with certainty to the other men. Fraction 1 is a very powerful immunogen capable of stimulating low mouse protective antibodies in individuals never exposed to plague antigen (C61, 62 and 63). This experiment on a small group of individuals reinforces observations made in 1951-1953 and discussed on pp. 18-19 of this report that Fraction 1, when used in proper dosage, may prove strongly immunogenic without causing side effects.

Final appraisal of clinical trial with 420,000,000 viable freeze-dried living attenuated EV (WR-S). Twenty-five vaccinated men developed pronounced erythema and some induration which lasted for 96 hours in 23 (92%); two had severe local reactions. All developed lymphadenopathies; six (24%) had fever, and seven (28%) disclosed a slight increase in WBC. It is apparent that these reactions were more severe than those produced by typhoid vaccine or U.S.P. Army plague vaccines. To some individuals, the effects of the EV (WR-S) were distinctly unpleasant. However, these effects were definitely milder than those produced by the 1951 EV 76 vaccine. Hemagglutinins, which declined by the 28th day or disappeared by the 179th day, developed in 23 of the men. No CFA were recorded. U.S.P. vaccine and Fraction 1 antigen as a booster inoculation recalled these antibodies. The average mouse protective antibodies declined from 16.5 to 14.3; only one man had a titer below 5. The EV (WR-S) of low immunogenic efficacy failed to enhance the antibodies in the inadequately sensitized human subjects. U.S.P. plague vaccine, and in particular Fraction 1, proved effective as booster inocula.

Published reports (Payne et al., 1956; Aleksandrov et al., 1963; /Pollitzer, 1966, pp. 240-241/; and Girard, 1963) document the fact that single vaccinations with live EV 76 give demonstrable F_1 antibodies in only a few persons, while repeated vaccinations give persistent in vitro demonstrable antibodies. It is generally overlooked that the passive mouse protection test as originally described (Meyer & Foster, 1948) allows a more accurate assessment of immunity to plague by humoral antibodies than in vitro measurement of F_1 HA and CF antibodies. Modifications of the seroprotection test by Favarel (1949) using, according to Girard, "a more severe challenge than the standard", or a micro-quantitative procedure (Burrows, 1955) afforded meaningless figures. From the studies by Spivak et al. (1958) and many additional experimental plague infection tests on guinea pigs, mice and subhuman primates, it has been concluded that MP indices below 5 are fairly reliable indicators of immunity to infection. There is increasing evidence that the immunogenicity of a vaccine for man may be deduced from his serologic response. The argument that the ability of sera from vaccinated subjects to protect mice passively against challenge may provide information only on the levels of circulating antibodies protective for these animals may or may not be valid. It is not known that they are protective for man. Until this point is clarified, conclusions should be formulated only with reservations. However, since freeze-dried EV (WR-S) vaccine in the total dosage of 2 billion with 420,000,000 viable P. pestis failed to stimulate significant circulating protective antibodies in man, an explanation must be sought. The particular EV vaccine containing viable P. pestis behaves like a dead vaccine; it merely stimulates the appearance of HA antibodies and only 4% MP indices below 10. Since it fails to sensitize the recipient to a booster inoculation with the same vaccine, its immunogenicity is either inherently of a low order or may have deteriorated following freeze-drying.

Pilot tests on guinea pigs indicate that the Saigon EV isolate is deficient, regardless of the fact that it possesses all the biochemical and nutritional requirements of an attenuated P. pestis. The invasive ability or "residual virulence" in guinea pigs in comparison with the classical EV 76 of Girard and Robic is low. However, the vaccine confers protection upon this species in relatively small doses and upon langurs in large doses. According to these standards, generally accepted as valid, EV (WR-S) could be considered an effective live vaccine for human use. Since a very low percentage of human subjects develop mouse protective antibodies inferentially considered adequate to confer immunity against plague infection, it was decided to discontinue further experimentation until the complex factors responsible for these disparities are understood.

III. Protection conferred by EV (WR-S) upon subhuman primates.

(A) Clinical reactions, serologic response and protective efficacy of freeze-dried living P. pestis EV (WR-S) against experimental plague infection in Hanuman langurs (Presbytis entellus).

The first pilot experiment to appraise the protective efficacy of the freeze-dried living P. pestis EV (WR-S) vaccine in Hanuman langurs was discussed in Report #17 (pp. 20-24). Groups of excellently conditioned Presbytis entellus, inoculated intradermally with dilutions of the reconstituted vaccine containing 17,800, 178,000 and 1,780,000 viable P. pestis EV (WR-S), responded vigorously with serum antibodies. But two langurs (Nos. 83 and 100 in Table 10), despite mouse protection indices below 5, and four others (Nos. 85, 132, 99 and 101) with MPI between 5 and 10, succumbed to septicemic plague following subcutaneous challenge with 232,000 virulent P. pestis 195/P 2 months and 19 days after the vaccine inoculation. The largest dose of vaccine (1,780,000 organisms) protected two out of three of the langurs; both had MPI below 5 (0, 4). Since a single dose of 400 viable P. pestis of the EV (WR-S) vaccine had completely protected guinea pigs, it was reasoned that a single inoculation would be fully adequate to insure total immunity in simian species. But the actual experiment did not support this concept. Experience had proved that two inoculations (the so-called booster schedule) enhance the protective efficacy of any dead vaccine. Two experiments, one with Macaca mulatta (described in Report #17, pp. 26-28) and one with Presbytis entellus (to be described), proved that this principle applies equally to live EV vaccines.

(1) Two-dose booster-effect inoculations of Hanuman langurs.

Dilutions similar to those used on human subjects as detailed in preceding sections of this report were made from the suspension of reconstituted freeze-dried living EV 76 (WR-S). Six langurs were each subcutaneously inoculated with 53,000,000 viable P. pestis in a total of 270 million, while one Presbytis received 5,300,000 plague bacilli. The inoculations were well tolerated; fleeting local erythema and indurations were noted in two animals. Antibody response, measured by a modified hemagglutination technique, was modest.

Antibodies rose to significant levels following the second inoculation (booster) of 4.45 million viable bacilli in a total of 30 million P. pestis EV (WR-S) on the 50th day after basic vaccination. The HA, CF titers and MPI recorded in the peripheral blood serum on the day (49th) after booster and on the day the langurs were subcutaneously infected with 420,000 virulent P. pestis 195/P, and their response to plague are listed in Table 7.

Differences in resistance to infection between animals receiving single vaccination and revaccinated animals were not striking. All

TABLE 7

Antibody Response in Langurs (_Presbytis entellus_) to Vaccination With Living EV (WR-S) Vaccine. I. 53.5 x 10^9; and II. 0.535 x 10^6. Four boostered with 4.45 million of same vaccine on day 49 after vaccination. Resistance to subcutaneous plague infection with 420,000 virulent _P. pestis_ 195/P on day 50 after booster, or 100 days after basic vaccination.

	Dosage and number of animal	28 days		49 days Post-booster			Temp	Blood Culture	Local Reaction	Resistance or day of death and autopsy	Serologic Reactions 36 days after challenge	
		HA	CF	HA	CF	MPI					HA	CF
I.	53.5 x 10^6											
	SE 208 no booster	4	0	256	0	4.0	+	-	LR,A	Survived	2,048	16
	SE 222 no booster	8	8	512	4	1.0	-	-	LR,L++	Survived	4,096	4
	SE 225 no booster	16	16	256	0	1.0	-	+	N,L	9th, secondary enteric infection, septicemia	-	-
	SE 213 booster	64	4	512	0	3.0	-	-	LR,LA	Survived	65,536	8
	SE 234 booster	0	0	128	0	-**	+	+	LR,LA	Survived	131,072	32
	SE 235 booster	0	0	16	0	14.0	+	-	LR,A	Survived	8,388,608	32
II.	0.535 x 10^6											
	SE 200 booster	512	16	256	0	3.0	-	-	LR,SA+	Survived	524,288	64
III.	Controls											
	SE 257	-	-	-	-	16	+	+	LR	5th, septicemia		
	SE 269	-	-	-	-	15	0	0	LR	4th, septicemia		
	SE 270	-	-	-	-	16	+	+	LR	5th, septicemia		

LR = local reaction; A = abscess; LA = lymphadenitis; SA = suppurating axillary lymphadenitis _P. pestis_;
** = inadequate amount of serum to determine MPI accurately.

controls succumbed on the fourth or fifth day with lesions and bacteriologic findings of acute septicemic plague. Only one langur (SE 225), visibly ill with P. pestis in the blood stream on the fifth day, died on the ninth day with gross lesions of toxemia and an overwhelming blood stream infection with enteric bacteria. Another reinoculated animal (SE 235) became clinically ill, had fever, and yielded a positive blood culture, but survived. The resistance conferred by the vaccination was not absolute. With the exception of SE 225, all animals developed local inflammatory lesions varying in diameter from 3 to 7 cm at the site of infection. The centers of the swellings in three became necrotic and the discharging pus contained virulent P. pestis. Lymphadenopathy accompanied the local plague lesions; in one langur, the right axillary lymph node had perforated by the 16th day. The degree of resistance is indirectly indicated in five animals with MPI below 5 (1.0 to 4.0). Two exceptions were noted: one langur with an MPI of 14 survived, while the other, with an MPI of 1.0 and apparently fatally weakened by the generalized infection following endotoxemia, succumbed to a secondary bacterial infection of the intestinal tract.

One observation deserves special comment: On recovery from plague, the five langurs had many more HA and CF antibodies by the 36th day than were obtained when they received only the vaccine. The significance of this vigorous antibody response to challenge will receive special attention in future protection tests with langurs.

This experiment fulfilled an important purpose. The EV (WR-S) vaccine in a dosage of 53,000,000 freeze-dried viable plague bacilli protected six out of seven langurs against fatal but not local plague infection. The only fatality had not received a booster, but two other non-boostered animals were protected. Immunity was indirectly reflected by the presence of significant protective antibodies in the peripheral blood. The same vaccine in identical dosages subcutaneously inoculated into human subjects failed to stimulate HA or CF and mouse protective antibodies following either basic or booster vaccination. Thus the prevailing concept that plague vaccines demonstrating a very good presumptive efficacy in mice, guinea pigs and moderately susceptible subhuman primates may be used to immunize man, must be accepted with definite reservations. It was reasoned that the disappointing immunogenic response of human subjects to intracutaneous (Walter Reed Army Institute of Research trial) or subcutaneous vaccination was attributable to low-dose inoculations. To test this concept, 25 human subjects were vaccinatedwith the standard dose as originally recommended by Girard (1935-36) and Korobkova (1939, 1956). A series of langurs were similarly inoculated with the same vaccine dilutions.

TABLE 8

Antibody Response of Langurs (*Presbytis entellus*) Vaccinated Subcutaneously with Living EV (WR-S). Challenge to be made on 16th month.

			Antibody Titers				
			28th day			133rd day	
	No.	Viable Cells	HA	CF	MPI	HA	CF
I.	1.	420 million	4,096	0	4	64	0
	2.	"	1,024	0	2	128	0
	3.	"	512	0	11	-	-
	4.	"	4,096	2	4	256	0
	5.		4,096	4	2	128	0
	6.	"	512	4	1	512	0
	7.	"	1,024	0	4	256	0
	8.	"	32,768	32	00	4,096	0
II.	9.	42 million	512	64	00	256	2
	10.	"	16,384	4	2	256	2
	11.	"	256	2	1	2,048	2
	12.	"	64	4	13	32	0
	13.	"	1,024	0	1	256	0
	14.	"	1,024	16	1	1,024	0
	15.	"	256	2	1	4,096	0
	16.	"	1,024	4	1	2,048	0

(2) Antibody response of Presbytis entellus vaccinated subcutaneously with 420,000,000 or 42,000,000 freeze-dried viable P. pestis EV (WR-S) in a total suspension of 200 or 2 billion plague bacilli.

Pertinent records of this experiment are summarized in Table 8. Both the 42 million and the 420 million total doses produced erythema, small nodules or edematous indurations extending over the femur for 72 to 96 hours, without impairment of function or general health. Enlargement of the lymph nodes could not be ascertained. Antibody response was of a high order, with HA titers reaching as high as 1:32,768. Even complement-fixation antibodies (titers 1:4 to 1:64) were present in eight animals. Except in two langurs (Nos. 196 and 207), the mouse protection indices were all below 5 on the 28th day after vaccination. The heterogeneous immune response of the 16 langurs was slightly more uniform but essentially similar to that succeeding the inoculation of dead vaccine. Since experience had demonstrated that langurs with high-level antibodies survived severe challenge infections, it was decided to apply this experiment to determine the duration of immunity. Two groups of langurs will be infected with virulent plague. One group will be challenged subcutaneously and the other by the aerosol route during the 16th month after vaccination. Before challenge some animals will receive a second inoculation of the original vaccine dose at a time interval similar to that selected in human mass vaccination.

(B) Susceptibility and immunity of Cercopithecus aethiops ((Linnaeus 1718) - grivet, vervet, African green monkey) to plague.

Intermittently shipped imports and tedious, expensive conditioning procedures have limited experimentation to relatively small groups of langurs, thus restricting the scale of experiments which may ultimately furnish meaningful conclusions. According to published records and inquiries made in South Africa, the plague susceptibility of the common African green or vervet monkey (Cercopithecus aethiops) has never been determined. Since this genus is readily obtainable, a few preliminary tests have been done.

(1) Susceptibility of Cercopithecus aethiops (from Ethiopia) to plague.

The susceptibility of Cercopithecus (from Ethiopia, weighing 2.4 to 4.9 kg) to subcutaneous plague infection was not uniform, but in general it was similar to that of langurs in individual resistance, course of infection, and time of death. Like langurs, individual Cercopithecus may succumb to 200 to 200,000 P. pestis, or, when they survive (Nos. 115 and 124), develop significant antibodies 21 days after infection (Table 9).

TABLE 9

Susceptibility of *Cercopithecus aethiops* (Ethiopian origin) Monkeys to Subcutaneous Plague Infection

No.	Weight kg.	Dose of Viable Cells	S/T	Day of Death	Weight kg.	Pathology and heart blood culture(+)	21 days after infection HA	CF	MPI
CA 118	3.2	209	1/4	29	2.0	Subacute metastatic pneumonia, septicemia(+)	512	0	-*
CA 123									
CA 127	3.0			29	2.6	" " (+)	256	0	5
CA 128	4.1			9	3.8	Acute septicemia (+)			
CA 117	2.4	2,090	0/2	9	1.8	Acute septicemia (+)			
CA 121	4.4			5	4.7	" " (+)			
CA 112	4.5	20,900	1/3	8	4.5	Acute septicemia (+)			
CA 114	4.6			5	4.6	" " (+)			
CA 115	5.0				-	Survived	2096	1:8	4.0
CA 122	4.5	209,000	1/2	6	4.4	Acute septicemia (+)			
CA 124	4.6				-	Survived	2096	1:4++++	2
CA 113	5.2	2,090,000	0/3	4	5.0	Acute septicemia (+)			
CA 126	4.3			11	3.8	" " (+)			
CA 129	5.9			3	5.5	" " (+)			

*9 mice died overnight and 1 mouse died on the 6th day
AC - anticomplementary
S/T - Survivors/Total

The belated deaths of two Cercopitheci on the 29th day after infection were attributed to terminal septicemic plague complicated by right principal lobar, left upper lobar, and patchy metastatic pneumonia in the left principal lobe in CA 127. The left and right upper lobes in CA 128 were covered with fibrin studded with necrotic nodules surrounded by hemorrhagic lobular consolidation and resembled the pathologic changes of metastatic pulmonary plague observed in guinea pigs dying from the disease during the 6th-15th day after infection. The pilot susceptibility experiment encouraged the use of Cercopithecus species as a substitute for langurs. However, there were surprises ahead.

(2) Lack of antibody response and of resistance to plague infection in aethiops sp. from Kenya.

Cercopithecus aethiops was included in experimental trials to evaluate the immunogenic and protective efficacy of living EV (WR-S) on langurs and human subjects. At the time of the trial, the same vaccine suspension in doses of 53.5 million and 535,000 that was used to inoculate seven langurs was administered simultaneously to members of a small shipment of six Cercopitheci recently imported from Kenya and conditioned and held at the National Center for Primate Biology. The inoculations were well tolerated, but the antibody response on 8, 16, 28 and 50 days after vaccination was negligible (Table 10). Four subhuman primates of the experimental series received the usual booster inoculation (4.45 million organisms). On the 49th day after the booster, the day before challenge, peripheral blood sera yielded no significant antibodies. The average MPI remained at the level (16) measured before vaccination. The large infective (420,000 organisms) dose of virulent P. pestis was chosen intentionally, since one of two Cercopitheci from Ethiopia had resisted an infection with 209,000 virulent plague bacilli. The entire group of vaccinated aethiops succumbed to acute septicemic plague either shortly before or following the death of the only available control animal. The failure to respond to vaccination was surprising, since in a comparable experiment the EV (WR-S) vaccine had conferred good protection on langurs. Nothing is known concerning the capacity of Kenya vervets to respond to plague antigens or vaccines. On recovery by the 21st day after infection, the Ethiopian species had MPI below 5, and even complement-fixing antibodies. The Kenya Cercopithecus may differ in genetic make-up and prove more susceptible than the Ethiopian species. Negotiations are in process to repeat this type of experiment with large series of South African Cercopithecus aethiops pygerythrus. On first thought, such an investigation might appear non-essential to the task of developing an effective attenuated plague vaccine for man. But this quest could be considerably accelerated if a subhuman primate uniformly susceptible to plague infection with relatively few bacilli were readily available in adequate numbers for systematic clinical trials and protection tests.

Recent experiments introducing a new factor--a highly invasive EV 76 vaccine isolate for aethiops species--warrant a detailed report.

TABLE 10

Antibody Response of Kenya aethiops Group of Cercopithecus to Living EV (WR-S) Vaccine. Four boostered with 4.45 million total bacilli of same vaccine on 49th day after vaccination. Resistance to subcutaneous plague infection with 420,000 virulent P. pestis 195/P on 50th day after booster, or 100 days after basic vaccination.

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Dosage and Number of Animal	28th day HA	28th day CF	49th day Post-booster HA	49th day Post-booster CF	49th day Post-booster MPI	Temp	Blood culture	Local Reaction	Results of challenge Day of Death and Autopsy
I. 53.5 x 10^6									
CA 130 no booster	0	0	32	0	-	-	+	LR	6th; septicemia, secondary pneumonia
CA 133 no booster	0	0	8	0	15.0	+	+	LR	6th; septicemia
CA 135	4	0	4	AC	20.0	-	+	LR	3rd; septicemia
CA 136	64	0	-	-	-	-	-	-	
CA 137	64	0	8	0	16.0	+	+	LR	4th; septicemia
II. 0.535 x 10^6									
CA 134	8	0	4	0	16.0	+	+	LR	3rd; septicemia
CA 138	0	0	4	0	13.0	+	+	LR	4th; septicemia
CA 132 Control						+	+	LR	5th; septicemia

MPI - average 16 before vaccination

LR - Lymphadenitis and local inflammatory reaction

IV. Antibody Response and Protective Efficacy of a new EV vaccine designated EV 51f.

(1) Introduction: At a recent traveling seminar on plague control, the following qualifications for a living plague vaccine, given in translation from a paper presented in Russian, were offered for consideration:

> The plague vaccine strain must possess a complex of properties typical for *P. pestis*. The determinant characters must be F_1+, V&W+, Pu+ and P- and the vaccine must be safe for rodents and man. Fifteen billion (optical count) attenuated *P. pestis* inoculated subcutaneously may cause a one-fifth loss in weight by the sixth day after inoculation (i.e., loss in 1/5 of the animals inoculated is within experienced reason), as well as local abscesses and granulomas in liver and spleen, but no deaths must occur. The bacilli must retain "residual" virulence, which is manifested by the capacity to multiply and to persist ("implant") in the organs of the animals for a few days in order to produce reactive changes in the spleen and liver: i.e., proliferation of histocytes in the interalveolar septa, hyperplasia of the reticulo-endothelial elements, macrophage reactions, formation of plasma cells. At termination of the vaccination process, no organic pathologic changes should remain. Vaccine strains must immunize guinea pigs. Live vaccines must contain intact antigens potentially capable of producing *in vivo* the "missing" antigenic components not formed *in vitro*, which should "induce higher resistance to plague than killed or chemical vaccine" (Korobkova, 1965). The meaning of the last sentence is not clear, nor has it been clarified by the author.

In connection with the problems which arose during testing of the EV (WR-S) vaccine, the annex to the preceding definition warrants quotation:

> "Of great importance is the problem of stabilization of immunogenic properties of plague vaccine isolate. A strain can be stabilized for the longest period provided it is transferred into an anabiotic state by lyophilization. However, some vaccine strains even under such conditions of storage lose some immunogenicity. Repeated lyophilization has been found to reduce the effectiveness of the strain. Repeated passage of vaccine strains in animals, particularly in the presence of non-toxic doses of iron salts,are conducive to stabilization and enhancement of immunogenic properties of vaccine strains".

Since the viability (approximately 30%) remained constant for over two years, vaccination trials with freeze-dried EV (WR-S) plague vaccine (previously discussed) were undertaken. Guinea pigs, each subcutaneously vaccinated with 1,000,000 (10^6) viable organisms, were completely protected, and partial resistance was obtained with 2,000 viable plague bacilli. In one experiment, the 50% protective dose was less than 40 viable organisms. A more recent test demonstrated that a higher dose of 845 was required to protect half the mice, while 84,500 P. pestis were required to confer complete protection. Regardless of these qualifications, the freeze-dried attenuated P. pestis vaccine EV (WR-S) failed to stimulate significant blood serum antibodies in human subjects. Because of the statement of Professor E. I. Korobkova at the seminar on plague in Stavropol U.S.S.R. (1965) that EV 76 is the most stable and effective of all vaccine strains now used for immunoprophylaxis of plague, the warnings of Jawetz & Meyer (1944-1945), Chen & Meyer (1955) and in particular, Otten (1940) that the immunogenicity of different isolates from attenuated strains of P. pestis may differ strikingly for guinea pigs, mice and probably for man, were overlooked. Since active immunization with EV (WR-S) was accompanied by the production of circulating antibodies in guinea pigs and subhuman primates, it was anticipated that the vaccine would prove equally efficacious in man. This concept gained some support in a clinical trial with the EV (WR-S) isolate conducted in 1951 on serologically tested humans. Four, or 30%, of the 12 subjects vaccinated with transplants of EV 76 obtained from Madagascar (1951) developed MPI below 10 following subcutaneous inoculation of approximately 340,000,000 viable P. pestis in a 1 billion total.

Theoretically, the lack of antigenicity manifested by the EV (WR-S) vaccine may be attributed to some or all of the following unknown factors: (1) The original seed culture may have lost its early immunogenic efficacy due to improper cultivation and storage. An isolate of EV 76 obtained from the Lister Institute in 1939 (three years later) failed to produce the splenic reactions considered by Bablet and associates (1937) as a test for the antigenic value of a vaccine possessing "conservation of a certain degree of pathogenicity and virulence". (2) It is suspected that cultivation on blood and hormone agar, together with inadequate storage at ±4^{o}C, may have been responsible for the deterioration (see Girard, 1963, p. 67). (3) The percentage of virulent to low-virulent members of the P. pestis population may have shifted, for reasons not yet understood. The number of virulent, invasive plague bacilli apparently adequate for protection of guinea pigs, mice and langurs was not adequate for man. (4) In some unknown manner, freeze-drying of the EV (WR-S) isolate in 7.5% sucrose, 0.7% glutamate and 2.5% human serum albumin may have progressively altered the immunogenic efficacy of the vaccine. Girard (1963, p. 67), in view of observations made by Robic (1952), expressed reservations concerning lyophilization of EV vaccine preparations. He stresses the aspect of viability, but not changes in immunogenicity. To clarify some of these influential factors, a systematic study was initiated.

The observations of Walker et al. (1953), fully confirmed by others (cf. Girard, 1958), established the fact that living vaccine strains, and in particular EV, can multiply appreciably in mice and guinea pigs, although such multiplication is controlled before it reaches serious proportions. In a follow-up study, Payne et al. (1955) demonstrated that cortisone greatly enhanced the "virulence" of EV, but that differences between "virulence" and "avirulence" in strains of P. pestis became indistinct. While these studies were in progress, Jackson & Burrows (1956) and Avanyan & Gubina (J. Microbiol. Epidemiol. Immunol., 1961) achieved an enhancement of virulence. When they injected low-virulent strains of P. pestis into mice or guinea pigs treated with ferrous sulfate (equivalent to 25 ug Fe/gm body weight), they observed that the whitish or straw-colored P- colonies formed on a particular defined medium containing haemin--characteristic for avirulent strains--reverted to densely pigmented P+ colonies after animal passage when the strain had acquired virulence. The possibility of maintaining, restoring, or increasing the immunogenicity of live attenuated EV by animal passage has been tested by Soviet workers (Levi et al., J. Microbiol. Epidemiol. Immunol. 31:1512, 1960). In a very careful study they passaged a vaccine strain in white mice and conclusively demonstrated increased multiplication and antigenicity in relation to this animal; but no increase in the virulence of the vaccine strain was observed in the course of 32 continuous passages. The number of bacilli--58--which after the 31st passage proved capable of immunizing 50% of the animals contrasts with the 33,000 bacilli originally required to give comparable protection. An increase in the antigenicity of the vaccine was accepted as proven. The authors have no explanation for this enhanced immunogenicity, but are inclined to suspect the selection of "more antigenic variants" during the process of passaging. The genotypic characters of the vaccine strain were not recorded, and it is not known whether this strain resembled the attenuated EV biologically defined as F_1+, V&W+, Pu+, and P-. The studies of Levi et al. were made on mice with Strain 1, an oceanic variety widely used as a vaccine strain in prophylactic immunization against plague. In response to the question, Did the virulence of the bacilli to white mice increase as the ability to multiply and the antigenicity of the adapted variant became enhanced?, they answered as follows: "The researches of a number of workers have shown quite convincingly that multiple passaging of the vaccine strains used for the specific prophylaxis of plague does not cause them to revert, i.e., to increase in virulence." Results of their experiments agreed with this statement. However, conclusions are based on pathogenicity tests, which are not conclusive. Graded doses of plague bacilli suspensions derived from different passage lines were injected subcutaneously into 10-20 white mice in each experimental group, and the mortalities were recorded for three to four weeks whether or not plague bacilli were isolated from the mice. The LD_{50} and standard deviations were calculated. The investigators claim that the slight differences in the LD_{50} were not statistically significant. Without quantitative bacteriologic examinations of the heart blood of the inoculated mice, these conclusions must be accepted with reservations.

Plans were made to study the various changes in virulence and immunogenicity of an EV 76 isolate after passage through guinea pigs. In searching for an EV isolate suitable for such a study, several lyophilized transplants maintained in our collection were sampled in pilot tests on guinea pigs. Observations disclosed that the original transplant brought to the U.S.A. from Madagascar on April 12, 1951 by the late Dr. Kenneth Goodner possesses characters previously unrecognized. The history of this EV strain 51 from Madagascar, available for detailed study, is as follows:

On May 10, 1951, a mass culture prepared from the original slant was grown on hormone agar at 37°C for 48 hours. Suspensions prepared in a stabilizing solution consisting of lactose-salts solution in the proportions found in skim milk were lyophilized by Cutter Laboratories in vials sealed under dry nitrogen and stored at -10°C (Quan, Chen and Meyer, Bacteriologic Proceedings, Abstract M46, p. 78, 1956). The viability determined for randomly selected vials varied from 35-65%. Little viability was lost in five years. The PD_{50} in mice for the fresh suspension was 3,500 viable organisms; after freeze-drying, it was 11,000. Only one vial was opened in 1965 for toxin studies.

For the purpose of stabilization and enhancement of the immunogenic efficacy, the EV 76 freeze-dried in 1951 was repeatedly passaged through guinea pigs previously inoculated with nontoxic doses of iron salts. One of the last two available ampoules of lyophilized EV 51 was opened on August 25, 1967. The reconstituted contents were grown on a blood agar slant at 37°C. A guinea pig given $FeSO_4$ in oil intramuscularly was inoculated with 0.5 ml of a 24-hr. 37°C-grown broth culture (approx. 100 million viable organisms) seeded with the growth from this slant. The animal was sacrificed on August 31, 1967, and the emulsified spleen was inoculated into a guinea pig pre-treated with $FeSO_4$. Three additional spleen passages through treated guinea pigs were followed by the inoculation of an untreated guinea pig. From the enlarged spleen, studded with granulomas, a blood plate yielded numerous typical smooth plague colonies. Ten were picked from separate blood agar slants. All except one grew, and on immunogenicity tests with viable subcutaneous dosages ranging from 3000-6000, protected mice as follows: five colonies protected 100%, three - 90%, and one - 70%. A potency test made on the slant culture on November 22, 1967 yielded a PD_{50} of 3,600 in mice. This culture, known as attenuated viable P. pestis EV 76 51f, has been tested as follows:

A. Protective efficacy of 37°C 48-hr. agar-grown suspension in guinea pigs subcutaneously immunized against standard subcutaneous challenge 28 days later:

Viable Dosage	S/T	
1.6	2/6	
16.8	5/6	
168	6/6	
1,680	5/6	PD_{50} = 5 organisms
16,800	6/6	
168,000	6/6	

Concurrently, the same inoculum was subcutaneously titrated in mice, which were challenged subcutaneously 14 days later. The 50% protective dose for mice was 1,100 viable organisms.

(2) Reactions of guinea pigs to EV 51f.

A titration was performed in guinea pigs to test the acute toxicity and lethality due to invasiveness following intraperitoneal injection of a 37°C, 72-hour agar-grown suspension. Animals dying in less than 72 hours post-vaccination were considered to have succumbed to immediate toxic effects of the inoculum, whereas animals dying after 72 hours were considered to have died from a combination of invasiveness and toxicity of the inoculum:

Viable Count Injected	Early Deaths 0-72 hrs.	S/T	Late Deaths	Total S/T
10.1 billion	5	0/5	-	0/5
101 million	0	5/5	2	3/5
1.01 million	0	5/5	0	5/5
10.0 thousand	0	5/5	0	5/5
101	0	5/5	0	5/5

Of the five animals expiring within 72 hours of inoculation, two died on the first day and the remaining three on the second day. Post-mortem examination confirmed the deaths as due to active toxicity of the inoculum; all five animals manifested fibrinopurulent peritonitis with hemorrhages and severe injection of the capillaries. The two guinea pigs dying late after receiving 101 million EV 76 51f exhibited the following changes:

No. 7784 died on the 10th day. Post-mortem examination revealed moderate peritonitis with blood-tinged fluid; an enlarged spleen, not necrotic; and mottling of the liver with fibrin deposits.

No. 7780 died on the 11th day. Post-mortem examination revealed emaciation, but little residual peritonitis; moderate injection of the subcutaneous capillaries; and an extremely enlarged spleen studded with innumerable granulomas. The liver was likewise enlarged, with similar foci and surface fibrin deposition. The lungs displayed

TABLE 11 - PART 1

Intraperitoneal Titration of 37°C, 72-hour HIV Agar-Grown Suspensions of Various EV Isolates in Guinea Pigs, and Results of Intraperitoneal Challenge with 4,500 Virulent 195/P 1 month later.

	Deaths due to Immunization		Results of Challenge	
EV 76 (Paris)	Toxic deaths 0-72 hours	Late Deaths	S/T	Average Day of Death
2.2 billion	3	0	a	-
22 million	0	0	5/5	-
220 thousand	0	0	5/5	-
2.2 thousand	0	0	5/5	-
22	0	0	4/5	6
MPD - 2.2 thousand. PD$_{50}$ - indeterminant, less than 22 organisms				
EV 76 (Devignat)				
3.25 billion	3	0	a	-
32.5 million	0	0	5/5	-
325 thousand	0	0	5/5	-
3.25 thousand	0	0	4/5	4
32.5	0	0	1/5	3.7
MPD - 325 thousand organisms. PD$_{50}$ - 325 organisms				
EV 76 (WR-Saigon)				
1.7 billion	2	1	a	-
17 million	0	0	5/5	-
170 thousand	0	0	5/5	-
1.7 thousand	0	0	3/5	4
17	0	0	1/6	5.5
MPD - 170 thousand organisms. PD$_{50}$ - 520 organisms				

TABLE 11 - PART 2

Protective Efficacy of EV 76 (Paris) by Intracutaneous and Subcutaneous Immunization Against Subcutaneous Challenge 28 Days After Primary Inoculation.

Intracutaneous S/T	Viable Count	Subcutaneous S/T
0/6	1	0/6
1/6	10	1/6
5/6	100	5/6
5/6	1,000	5/6
6/6	10,000	6/6
6/6	100,000	6/6

EV 76 (Paris) in mice, concurrent titration: The mouse PD_{50} is 2,000 viable organisms when challenged subcutaneously 14 days later.

a = animals surviving immunization, sacrificed and examined 18-20 days post immunization. In no case was any residual pathology seen.

All animals dying within 0-72 hours showed typical signs of toxic death on post-mortem examination. The one late death with 1.7 billion EV 76 (WR-Saigon), died 11 days post-immunization and also showed acute toxic manifestations with residual peritonitis. The spleen was only slightly enlarged and free from granulomas.

consolidation along the margin of the right lobe--an area of metastatic pneumonia.

The three remaining animals in this dosage group were consequently sacrificed and examined at 14 days post-vaccination. One of these was ill and emaciated at this time and had a necrotic and enlarged spleen similar to that of No. 7780. The liver of this animal was less involved, but both kidneys had suspiciously protruding granulomas. The lung exhibited one small pulmonary nodule. There was positive recovery of EV 76 51f from the spleen, liver and kidney. The other two animals manifested no residual pathology except for fibrinous adhesions between the liver and diaphragm.

The remaining survivors of this series were challenged intraperitoneally with virulent P. pestis at one month:

Viable Dose	Number of Animals Challenged	S/T	Average Day of Death
1.01 million	5	5/5	-
10,100	4	3/4	3
101	5	2/5	4.5

PD_{50} intraperitoneally = 1,010 P. pestis EV 51f

MPD = 1.01 million

For comparison, a similar intraperitoneal titration was performed in guinea pigs with agar-grown cultures from other EV isolates being considered for use. The data are presented in Table 11.

(3) Vaccination of Cercopithecus aethiops subspecies?) of Ethiopian origin. Vaccination fatalities.

Essential details of this unexpected and consequently important EV 51f vaccination study, followed by a protection test with virulent P. pestis, are summarized in Table 12.

Male vervet monkeys imported from Ethiopia were chosen, since pilot experiments had indicated that this genus is probably slightly more susceptible than langurs (Presbytis entellus). The monkeys were subcutaneously inoculated with saline suspensions of the isolate grown at 37°C for 48 hours on HIV* agar, in an area over the left femoral muscle permitting frequent examinations for local reactions with the smallest possible amount of handling. Only the large dose of 160.5 million viable organisms produced local reactions in the form of erythema and indurations. But most surprising was the fact that all the subhuman primates of Groups I, II and III became visibly ill on the third to fourth day and were listless, with glassy eyes, photophobia, anorexia, and loss of weight for eight days or longer.

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*Beefheart infusion broth with calcium, magnesium and xylose (Eisler).

TABLE 12 - PART 1

Clinical and Serologic Response to Vaccination with EV 51f and Resistance of Cercopithecus aethiops from Ethiopia to Challenge with 132,000 Virulent 195/P Subcutaneously on Xiphoid 41 days later (May 3, 1968).

Animal No.	16 days				28 days			Local Reaction to Challenge	Outcome
	a HA	b HA	CF	MPI	b HA	CF	MPI		
Group I:	160.05 million viable								
CA 163	2048	2048	16	00	256	32	00	Sl. H., no oed.	Survived
CA 149	2048	2048	AC	23	1024	AC	3	None	"
CA 158	512	1024	AC	16	1024	16	5	Sl. H., + ind. desc., no oed.	"
CA 152	died from vaccine inoculation on 5th day; no sera available.								
Group II:	16.05 million viable								
CA 154	128	512	4	-	1024	AC	1	None	Survived
CA 151	256	1024	2	-	Sacrificed while moribund on 18th day.				
CA 153	512	512	0	-	1024	AC	10	Sl. H., no oed.	Survived
CA 147	256	1024	AC	4	512	0	3	Sl. H., no oed.	"
Group III:	1.605 million viable								
CA 164	4096	4096	16	00	512	8	2	None	Survived
CA 155	16,392	16,392	4	00	512	8	1	None	"
CA 159	1024	4096	AC	00	0	4	2	None	"
CA 162	died from vaccine on 5th day.								

a = test with formalinzed RBC
b = normal test, regular RBC
H = Hemorrhage
oed. = oedema
ind. = induration
desc. = descending to abdomen.

TABLE 12 - PART 2

Clinical and Serologic Response to Vaccination with EV 51f and Resistance of _Cercopithecus_ _aethiops_ from _Ethiopia_ to Challenge with 132,000 Virulent 195/P Subcutaneously on Xiphoid 41 days later (May 3, 1968).

	16 days				28 days				
Animal No.	a HA	b HA	CF	MPI	b HA	CF	MPI	Local Reaction to Challenge	Outcome
Group IV: 160,500									
CA 157	4096	512	8	00	>8000	32	00	None	Survived
CA 160	1024	1024	AC	00	512	8	1	None	"
CA 161	32,768	1024	16	-	2048	32	00	None	"
CA 165	4096	4096	8	00	64	8	1	None	"
Group V: Saline Control Placebo									
CA 156	8	0	0	16	0	0	19	H & oed. desc. severe ind.	Died 5-6 day
CA 150	4	0	0	-	4	AC	16	"	Died 4 day
CA 166	4	0	0	-	0	AC	-	"	Died 4-5 day
CA 148	4	0	0	21	0	0	21	"	Died 6 day

a = test with formalinized RBC
b = normal test, regular RBC
H = Hemorrhage
oed. = oedema
ind. = induration
desc. = descending to abdomen

Two (CA 152 and CA 162) succumbed on the fifth and sixth day, respectively, to generalized septicemia with splenic tumor (42 gm, five times normal weight); while a third (CA 151), sacrificed while moribund on the 18th day, at autopsy revealed classical granulomas in the spleen and liver, originally considered by Girard as essential to the demonstration of immunogenicity of attenuated P. pestis EV 76. The splenic pulp of this aethiops monkey, when smeared over the surface of a blood plate, yielded 11 colonies which were phage-positive but nonpathogenic for mice upon intraperitoneal inoculation. According to Girard and Radaody-Ralarosy (1940), persistence of P. pestis EV 76 in guinea pigs inoculated intraperitoneally with one (1 millard) billion viable organisms never exceeded 13 days. The fact that in the highly susceptible Cercopithecus P. pestis EV 76 51f was present in the heart blood, spleen, liver, and bone marrow and persisted for at least five days longer following the inoculation of merely 16 million, offers impressive evidence that the attenuated P. pestis EV 51f is pathogenic in varying degrees for the Ethiopian species of Cercopithecus. Infective capacity or "virulence" is demonstrated by the proliferation and persistence of the vaccine strain in the affected tissues.. Among the host-effects, death was variable because the subhuman primates available were heterogeneous in their reactions. A graded dosage response was not measured. Even the low dose of 160,000 viable EV 51f organisms induced marked clinical manifestations of disease. No provision was made for measuring the host-effects more carefully except at autopsy. Visible lesions in the form of granulomas in the spleen and liver may be considered expressions of successful defense against a moderately virulent P. pestis. Of the parasite-effects, the most obvious was multiplication and survival of P. pestis in the tissues as an equivocal index of pathogenicity. Proliferation is a direct index of the extent and degree of parasitism. The effects of EV 51f in Cercopithecus were then compared with those observed in pathogenicity tests on Hartley guinea pigs. In the guinea pigs, the freeze-dried EV 51f in a total dose of 15 billion with 3 billion viable cells inoculated subcutaneously caused no deaths but resulted in local lesions at the site of injection and lymphadenopathy and granulomas in the spleen and liver. Thus, the isolate behaves like a moderately virulent microbe considered a safe vaccine for the immunization of man. Agar-grown suspensions at 37°C induce similar reactions in guinea pigs.

Table 8 demonstrates impressively that the antibody response was most vigorous in groups of Cercopitheci inoculated with the smaller doses of the vaccine strains. The Fraction 1 antibody titers were definitely higher in the sera of animals vaccinated with doses of 160,000 or 1,605 million. It is significant that on the 16th day after vaccination the mouse protection indices in these groups were already below 5. One serum dose of 0.5 ml protected all mice against the standard infection. This single observation lends support to the claim of those who have studied the immunity produced with P. pestis

EV 76 that by the fifth to sixth day after vaccination, definite signs of immunity are already demonstrable in guinea pigs. The slight fluctuations in titers are probably attributable to variable degrees of illness in individual animals. The pathophysiologic damage caused by the endotoxin released during the course of the vaccination disease may (if this is indeed the case) in some unknown manner interfere with the antibody-producing mechanism. This phenomenon was slight in Group IV, comprised of animals receiving the lowest dose of vaccine. It is not surprising that recovery from a moderately severe P. pestis vaccine infection should confer solid protection against subcutaneous infection with 130,000 or approximately 100 LD_{50} of virulent P. pestis 195/P. All the vaccinated Cercopitheci survived, while the unvaccinated controls inoculated with saline placebo died on the fourth to sixth day with clinical signs of apathy, anorexia, and fever, and positive blood culture on the third day, classical pathology and bacteriological evidence of plague septicemia at autopsy. It was intriguing that the animals vaccinated with small doses of EV 51f effectively managed the challenge inoculation. None developed local lesions or lymphadenopathy; two had moderate rises in temperature on the third day. Four to six monkeys vaccinated with large doses developed local subcutaneous hemorrhages faintly outlined in the skin but of relatively short duration, in one animal accompanied by induration and spreading edema. Release of endotoxin and hypersensitivity to Fraction 1 are generally connected with such local reactions. Why these lesions appeared only in some Cercopitheci vaccinated with large doses is a subject for further inquiry.

A mortality of 3 from a group of 16 Cercopitheci vaccinated with a generally accepted, "safe", attenuated live plague vaccine arouses concern and merits comment. The species of subhuman primates imported from Ethiopia has been superficially studied for susceptibility to virulent plague. The early deaths following subcutaneous virulent infection were characterized by ecchymotic hemorrhages within the skin extending subcutaneously with edema to the regional lymph nodes, which were invariably enlarged and hemorrhagic. All subcutaneous capillaries were distended and resembled the type of subcutaneous congestion commonly seen in rats dead from acute plague. Enlargement of the spleen and degenerative changes in the liver and kidneys, clear pleural effusions, and pulmonary edema and occasional petechiae were typical for acute plague. In contrast, autopsies on Cercopitheci dead from generalized EV 51f septicemia revealed the usual capillary ramifications in the dehydrated subcutaneous tissues, but no hemorrhages, necrosis or edema. The lymph nodes draining the site of inoculation were enlarged (the size of a Boston bean); they were merely hyperemic and mottled, not hemorrhagic. Spleens were invariably enlarged, in one instance five times the normal weight (42 gm). Granulomatous proliferations throughout the pulp, already present on the fifth and sixth days after vaccination, were extremely pronounced by the 18th day. Similar localized lesions appeared in the liver; they have not been observed in acute virulent plague septicemia. The kidneys of the vaccinated animals invariably exhibited evidence of an acute parenchymatous degeneration similar to that of plague. The microscopic changes essentially consisted of a degeneration of tubular epithelium with granular casts. Detailed examinations were not exhaustive, but the alterations resembled those which Walker (1968) ascribed to pathologic effects of the plague endotoxin. Varying (8 to 42 ml) amounts of clear transudate fluid in the thoracic cavities and pulmonary edema in both vaccinated and nonvaccinated subhuman primates may be attributed to the action of permeability-increasing plague toxins on vascular endothelium. The difference in pathologic changes observed in Cercopitheci which succumbed following vaccination, in contrast to those of fully virulent plague, was expressed by the absence of hemorrhages. Possibly this difference may be due to lower concentration of the causative toxins or to variations in the toxins causing the vascular damage. Further studies on subhuman primates may disclose the answer to the pertinent question: How injurious are attenuated living P. pestis EV vaccines to man? One such experiment now in progress is herewith detailed.

(4) Vaccination of green vervet monkeys (Cercopithecus aethiops subsp. ?) of Kenya origin. High vaccination mortality.

Male Cercopitheci, originally imported from Kenya, fully conditioned at the National Center for Primate Biology and intended for detailed susceptibility tests, were available. They were selected because a protective potency vaccination trial on this species from Kenya had, for unexplained reasons, failed. (See this report, Cercopithecus aethiops (2) Lack of antibody response and of resistance to plague infection, p. 48).

Five groups of four highly aggressive male Cercopitheci from Kenya, weighing 4.7 to 6.6 kg, and held in single cages after bleeding, were inoculated subcutaneously over the left femoral muscle with single doses of 0.5 ml of graded dilutions containing 218 million to 218 reconstituted freeze-dried living P. pestis EV 76 51f (Table 13). Within 24 hours, the majority of the animals in groups vaccinated with the larger doses of vaccine were less active, became more docile and ate sparingly. As the illness induced by the vaccine progressed, fevers with temperatures up to 41.2°C were recorded. Only the large doses resulted in local indurated swellings with or without involvement of local lymph nodes. The protocol stipulated the recording of daily WBC and quantitative blood cultures, but by the sixth day continuous handling had definitely aggravated the illness. Blood collections became increasingly difficult due to peripheral collapse of the veins and rapid clotting, and examinations were discontinued. On the sixth day after vaccination with 2.18 million viable EV 51f Cercopithecus No. 190 was found dead, with advanced rigor mortis. Autopsy of the dehydrated carcass revealed gross lesions of subcutaneous congestion of the skin, pleural effusion (28 and 40 ml), hypostatic lung edema, dilation of the heart cavities, splenic tumor, and the degenerative changes in liver and kidney previously encountered and interpreted as plague vaccine toxemia, without the hemorrhagic-edematous infiltration at the vaccination site. The left inguinal lymph node, though enlarged to the size of a bean, was soft, edematous, and hyperemic but not hemorrhagic. The intestinal tract contained little greenish chyme and few rectal pellets. Blood cultures on the fourth and fifth days after vaccination yielded confluent colony growth of EV 51f on blood plates. However, isolation and quantitative estimation of plague bacilli in cultures taken at autopsy were unsuccessful, since the organisms were apparently overgrown by enteric bacteria (E. coli, Proteus sp.). It is anticipated that, with the aid of selective media, isolation from the frozen tissues will prove successful.

In rapid succession, on the seventh day, three Cercopitheci (CA 174, CA 176, CA 183), and another three animals on the eighth day (CA 181, CA 179, CA 180), clinically ill with and without fever, with high WBC and blood stream invasion of varying intensity, came to autopsy. Gross pathologic findings were essentially the same as in the first victim of EV 51f vaccine. Enlargement of the regional inguinal and iliac lymph nodes, the size of the spleen, and the degree of pulmonary hypostasis were subject to individual variations. Secondary terminal bacterial invasion from the intestinal tract complicated the quantitative estimation of vaccine plague bacilli in the viscera. However, it is significant that at autopsy the heart blood cultures of four animals (CA 174, 176, 179, 180) yielded P. pestis EV on the surface of blood plates.

Two Cercopitheci (CA 191, CA 185) vaccinated with 218 million bacilli, and one (CA 189) inoculated with 2,180 EV 51f, with bacteriologically proven bacteremia, continued to lose weight despite careful nursing and died on the 15th, 20th and 28th day, respectively.

The group of four Cercopitheci each vaccinated with 218 viable organisms in an approximate total of 700 EV 51f appeared well, with no local reactions, only slight rises in peripheral leucocytes, and blood cultures repeatedly proven sterile. On the 28th day after vaccination, their blood sera reacted strongly to the passive hemagglutination test (1:1024 to 1:8192) and similarly to the CF test. The unexplained phenomenon observed in the first vaccination trial, i.e., animals vaccinated with small vaccine doses responded with slightly higher serum antibodies, was again evident.

The reactive behavior of this group is important because it indicates that a relatively small dose of live vaccine, provided it contains potentially invasive units, may effectively immunize Cercopitheci weighing from 5.1 to 6.6 kg without inducing local or systemic side-effects. Indirect evidence of protective efficacy has been confirmed: Cercopitheci surviving vaccination with EV 51f resisted subcutaneous plague with 100,000 P. pestis 195/P, which was fatal to nonvaccinated animals on the third to fourth day after infection.

Experimentation on aethiops sp. subhuman primates is full of surprises, but the outcome of the first experiment warrants a tentative conclusion. It is appropriate to emphasize the obvious fact that these Kenya Cercopitheci seem highly susceptible to the viable vaccine strain of P. pestis and its toxins. The infection causes severe side-effects, anorexia and pathophysiologic changes which prepare the host for secondary bacterial invasion from the intestinal tract. In retrospect, it is probable that the hard pellet food and frequent daily handling may have contributed to a high mortality. More elaborate care might have saved some of the experimental animals. One fact is paramount: no collection of animal hosts, however carefully selected, is so homogeneous in its reaction to infection that all individuals survive a given dose of microbes and all die after a slightly larger dose. It is apparent that currently available Kenya Cercopitheci are too heterogeneous for studies with small groups of animals. Plans are being formed and negotiated to repeat the experiment in South Africa with C. aethiops pygerythrus, using several hundred individuals, provided facilities can be procured. Further experimentation to test this subspecies for response to nonliving plague and other bacterial endotoxins is contemplated.

It is regrettable that the blood cultures could not be continued, but the data in Table 9 lend some support to the view that it was not the inherent toxic content of the vaccine which caused the mortalities. During the first six days after vaccination, surviving Cercopitheci furnished either sterile blood cultures, or else the bacteremia expressed in quantitative colony counts was of a low order. Thus it is believed that the fatal toxemia developed as a result of extensive proliferation of the vaccine plague strain and the continuous freeing of toxins which anchored to unaltered erythrocytes or endothelial cells, leading to that toxemia which is sensu strictu responsible for the death or preparation of the host for secondary bacterial invasion. These views are in agreement with those of R. V. Walker (Current Topics in Microbiology and Immunology, Vol. 41:23-42, 1967), who, on the basis of his research, speculated on the nature and mechanism of plague intoxications. The experiment just reported may serve to guide future studies evaluating the safety and protective efficacy of the classical living P. pestis EV 76 vaccine. Since EV 51f was reconstituted from the freeze-dried state, it is apparent that lyophilization and storage at -20°C for at least three months have not damaged or reduced the protective potency of the isolate. Although a different subspecies of Cercopithecus was used as experimental host for testing the immunogenic potency of the

TABLE 13

Clinical and Bacteriologic Response and Autopsy Findings in Cercopithecus aethiops (subspecies?) from Kenya, Vaccinated Subcutaneously with Freeze-Dried Living Attenuated EV 76 51f.

		Systemic Reaction		Blood culture^{xx} hours								Antibody response of survivors 28th day		
No.	Vaccine Dosage	Fever*	WBC	48	96	120	144	Day	Hbl	Spl	Lng	HA	CF	MPI
CA 173	218 million	40.5°	17,800	0	96	88	+	-	-	-	-	2048	4	-
CA 174	"	39.7	28,300	0	I	C	C	7	+	?C	+	-	-	-
CA 185	"	40.3	11,500	0	28	24	ND	20	+	+	+	-	-	-
CA 191	"	40.5	24,500	0	76	200	+	15	?	?	?C	-	-	-
CA 176	2.18 million	40.2	66,900	0	C	ND	+	7	+	+	+	-	-	-
CA 178	"	41.2	17,700	10	+C	+C	++C	-	-	-	-	2048	>16	-
CA 181	"	41.3	51,900	0	C	C	C	8	?	+	?C	-	-	-
CA 190	"	40.9	25,600	-	C	C	-	6	?	?	?C	-	-	-
CA 175	218,000	40.4	16,700	0	0	0	0	-	-	-	-	2048	AC	-
CA 179	"	38.9	44,900	0	C	?	+	8	+C	+C	-	-	-	-
CA 183	"	40.1	16,000	0	C	C	C	7	?	?	?C	-	-	-
CA 187	"	40.4	18,300	-	6	1	0	-	-	-	-	4096	2	-
CA 177	2,180	38.4	21,700	0	0	+	+	-	-	-	-	512	8	-
CA 180	"	39.2	38,800	-	20	100	+	8	+	+	-C	-	-	-
CA 182	"	38.2	17,900	-	40	0	0	-	-	-	-	4096	>16	-
CA 189	"	38.2	23,100	-	0	+	-	28	0	+	-C	-	-	-
CA 184	218	39.4	16,000	-	0	0	0	-	-	-	-	1024	2	-
CA 186	"	39.0	10,900	-	0	0	0	-	-	-	-	8192	>16	-
CA 188	"	38.4	14,000	-	0	0	0	-	-	-	-	8192	8	-
CA 192	"	38.0	11,100	-	0	0	0	-	-	-	-	1024	4	-

AC - anticomplementary
E.I. - erythematous indurated areas; oedema (cm)
xx - blood culture, number of colonies in 1 ml
WBC - highest white blood count

C - confluent growth (?contamination)
* - Fever, highest temperature
L - lymphadenopathy, degree +

ND - not tested
No L - no local reaction

freeze-dried preparation, the response was of the same magnitude as that seen in the Ethiopian animals which were given the aqueous suspension of the EV 51f isolate grown on agar at 37°C.

The following immediate studies are proposed:

(1) A comparative test to determine the stability of the freeze-dried living EV 51f at frequent intervals.

(2) Since in order to avoid unpleasant local reactions Soviet immunologists recommend the cutaneous or scarification method of vaccination with living plague vaccines, it is advisable to titrate and determine the minimum vaccine dose of EV 51f required to stimulate significant mouse protection indices on human subjects.

(3) The Commission on Immunization should discuss the feasibility and value of such studies.

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13. ABSTRACT

In a clinical trial on 25 human subjects each inoculated intramuscularly with 1 ml of dead U.S.P. plague vaccine (E medium) in accordance with the newly issued directions for military use, the preparation caused mild local and systemic reactions. The basic inoculation reduced the mouse protection indices to below 10 in 16% and below 5 in 4% by the 28th day, and thus conferred little or no immunity. However, a small booster inoculation of 0.2 ml brought the MPI to below 10 in 65% and below 5 in 26% of the human subjects. The U.S.P. plague vaccine in two inoculations three months apart protected 66% of Hanuman langurs against fatal plague, provided the MPI were low.

A freeze-dried attenuated living, slightly invasive EV (WR-S) vaccine proved immunogenic and protective for mice, guinea pigs and langurs. In a clinical trial on 25 men each inoculated subcutaneously with 420,000,000 viable organisms in a total of 2 billion P. pestis, this living vaccine produced unpleasant local and systemic reactions but failed to stimulate significant MPI antibodies or to confer the potential immunity-priming action generally observed with the U.S.P. dead plague vaccine.

An attenuated living EV 76 culture obtained from Madagascar in 1951, after passage through guinea pigs pretreated with iron sulfate considered "safe" according to international standards, proved highly protective for mice, guinea pigs and African green monkeys (Cercopithecus aethiops). However, in doses as low in concentration as 2,180 viable organisms, it induced 50% vaccination fatalities in Cercopitheci from Kenya and 15% in the Ethiopian species. This EV 51f strain in a dose of 218 organisms stimulated low MPI antibodies and conferred upon these highly susceptible species of subhuman primate a solid immunity against severe plague infection.

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U.S.P. dead plague vaccine grown on E medium, commercially manufactured by Cutter Laboratories. Antibody response in human subjects expressed by mouse protection indices. Protection conferred by this vaccine on subhuman primates (*Presbytis entellus*)						
Freeze-dried attenuated living *P. pestis* EV (WR-S) vaccine Efficacy in *Presbytis entellus* In dosages of 420,000,000 viable organisms induces local and systemic reactions, but no significant antibodies or potential immunity.						
EV 51f, a living attenuated plague strain highly immunogenic and "safe" for guinea pigs, produced vaccination fatalities (50%) in African green monkeys (*Cercopithecus aethiops*)						

AD______________

STUDIES ON THE MECHANISMS OF HYPERSENSITIVITY PHENOMENA (U)

FINAL REPORT

by

Abraham G. Osler, Ph.D.

March 1968
(For the period 1 July 1963 to 30 June 1968)

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SUMMARY

The Progress Report which follows summarizes two studies. The first deals with experimental infection of the rat with *Plasmodium berghei*. These studies include the use of an electronic particle counter to estimate the degree of parasitemia in the infected rat, and the development of an assay procedure for the protective action of humoral antibody. Indirect evidence has also been obtained which points to the merozoite or trophozite as the developmental forms which react with the antibody that suppresses or prevents infection with *P. berghei*.

The second investigation is a continuation of this laboratory's interest in mechanisms of hypersensitivity. *In vitro* studies with rabbit platelets and rabbit leukocytes point to the existence of two different classes of immunoglobulins capable of mediating the release of vasoactive compounds. One immunoglobulin which is reactive with the platelet is present in hyperimmune sera. It does not bind to the platelet as a prelude to interaction with antigen. The immunoglobulin which releases histamine from washed suspensions of rabbit leukocytes is bound to these cells and appears during the early stages of immunization. It may be the same immunoglobulin which mediates PCA in the rabbit, as described by Becker and his colleagues. The platelet and leukocyte reaction systems also differ in their requirement for plasma. The plasma requirement is only demonstrable in the allergic response of rabbit platelets. Rabbit leukocytes respond to antigen in the absence of plasma in the fluid phase.

The research program supported by this grant has been devoted almost exclusively to studies aimed at characterizing the mechanisms of tissue damage associated with serum dependent allergic responses of the immediate type. An important segment of our effort has been applied to the development of theoretically-valid in vitro procedures for following the steps initiated by an immune event and culminating in the release of vasoactive compounds. Following the demonstration of serum complement involvement in the formation of anaphylatoxin (1-3), we turned to two cellular systems based on the allergic response of human leukocytes and rabbit platelets.

During the course of our investigations on hypersensitivity mechanisms with in vitro models, two studies on auto-immune disease were completed, dealing with systemic lupus erythematosus (4-6) and with rheumatoid arthritis (7-9). Most recently, and as a consequence of Dr. Carter L. Diggs' assignment to our laboratory, we have initiated an investigation dealing with humoral immunity in rodent malaria. These studies are currently being prepared for publication. A brief summary of our findings in each of these areas follows:

A. Humoral Immunity in Rodent Malaria. (With Dr. Carter L. Diggs)

This investigation was approached with the purpose of identifying an antibody function which would reflect the immune status of the host. Two experimental plans were devised, based on considerations of cellular and of humoral immunity.

1. Blood clearance experiments.

Hypersplenism and increased activity of the reticulo-endothelium system have often been considered as essential manifestations of resistance to reinfection with malaria parasites. This facet of the problem was studied in the following manner. Erythrocytes parasitized with *Plasmodium berghei* were purified by flotation of blood from infected rats on 25% bovine serum albumin. These parasitized basophilic erythrocytes (PBE) were harvested and washed, yielding suspensions that contained fewer than 10% of nonparasitized red cells. The PBE's were labeled with ^{51}Cr, washed again and injected into normal and malaria-convalescent Fischer rats. The animals were bled at repeated intervals during the next few hours (as in the technique described by Biozzi, Benacerraf, et al.), and the blood samples assayed for ^{51}Cr. Blood clearance curves were thus generated. With malaria-convalescent rats, the rate of blood clearance was generally twice as fast as that obtained in studies with normal rats. Passive transfer of serum from convalescent rats failed to augment the blood clearance rate in normal animals. These findings typify the situation generally associated with "acquired cellular resistance." The physiological significance of a two-fold increment in the rate of blood clearance emerges from the following calculations. Assuming a circulation time of 10 to 15 seconds, and a generation time of about 24 hours for P. berghei, an inoculum of about 10^6 PBE would disappear completely within 24 hours at the clearance rates observed in immune animals. Under identical conditions, the normal rat would harbor at least the same number of parasites.

Despite these considerations, further studies of blood clearance were discontinued since the two-fold increase in slope did not provide sufficient leeway for more detailed quantitative studies.

2. Passive protection with serum antibody.

On the basis of earlier reports in the rodent, monkey, and man a systematic study of the protective effect of serum antibody on P. berghei infections of the albino rat was also undertaken. We decided to concentrate our efforts on the early stages of infection and thereby reduce the far larger number of variables met with in the more chronic situation.

a. Estimation of parasitemia by electronic particle counting. This procedure was developed to facilitate the estimation of parasitemia in the infected animals. In brief, a measured volume of tail blood (5 $\mu\ell$) is added to 10 ml of a saponin-EDTA-NaCl diluent (100 μg per ml, 0.02 M and 0.125 M, respectively). The saponin lyses the erythrocytes enabling enumeration of plasmodiae and reticulocytes in the Coulter Counter equipped with a 70 μ aperture and 0.5 ml manometer volume. The threshold is adjusted to enumerate particles equal to or greater than 2-3 μ. The concentration of particles in whole blood is estimated from the relationship

$$P = \frac{D}{V} \left[\bar{C} + K \left(\frac{\bar{C}}{10^3} \right)^2 \right] - \bar{P}_n ,$$

P = Concentration of particles in whole blood per ml

D = Dilution factor

V = Volume of diluted sample counted, in ml

$\bar{C}$ = Mean Coulter Counter reading

$K \left(\frac{\bar{C}}{10^3} \right)^2$ = Coincident correction factor, which varies with the aperture and volume of diluted sample

$\bar{P}_n$ = Mean value of $\frac{D}{V} \left[\bar{C} + K \left(\frac{\bar{C}}{10^3} \right)^2 \right]$ for nonparasitized normal rat blood

The validity of the procedure has been extensively controlled with microscopic counts of fresh preparations and stained smears. The dilution of blood and machine settings have been so arranged as to yield a particle count of about 100 x 10^6 per ml of whole blood in a rat with a parasitemia of about 3%. The enhanced facility and precision of estimating parasitemia by this procedure is readily apparent.

b. Passive protection assays. These are carried out by injecting a mixture containing convalescent serum and a standard inoculum of 2.5×10^6 parasitized erythrocytes per 100 gm rat.

The rats are bled 3 days later and the extent of parasitemia assayed as indicated above. The protective effect of the serum is concentration-dependent, yielding complete suppression at low dilutions. Potencies of different sera can now be compared in terms of the 50% suppressive dose, SD_{50}.

c. Pertinent findings. Utilization of this relatively simple, yet sensitive antibody assay has provided the following information.

1. The assay is highly reproducible under standardized conditions.
2. Little, if any, protective activity has been demonstrated in the sera of animals immunized with nonviable plasmodiae.
3. The protective activity in convalescent sera is associated with the 7S serum fraction obtained by molecular sieve filtration on Sephadex G-200.
4. The protective activity cannot be absorbed with erythrocytes from noninfected animals, or with parasitized erythrocytes.
5. *In vivo* studies of antibody disappearance suggest that an extra-erythrocytic developmental form of *P. berghei* unites with the antibody. Preliminary experiments point to the merozoite or trophozite as the "protective antigen."
6. Passive immunization exerts therapeutic as well as prophylactic activity in the rat.

B. In Vitro Studies of Human Reaginic Allergy. (With Drs. Lawrence M. Lichtenstein and David A. Levy)

Dr. Lichtenstein with whom these studies were initiated has successfully completed the requirements for the Ph.D. degree in the Department of Microbiology, The Johns Hopkins University School of Medicine. Upon completion of the residency training program with the Department of Medicine, he accepted the appointment of Assistant Professor in the Department of Medicine at this university. Dr. Levy joined the Department of Radiological Sciences at this university as Assistant Professor.

Our work in this area has recently been summarized in Advances in Immunology, vol. 8 (10).

1. *In vitro* studies of histamine release with human leukocytes from allergic donors.

A reaction system comprising leukocytes isolated from the peripheral blood of allergic donors, suspended in a tris buffer containing albumin, calcium and magnesium, and a highly purified ragweed pollen extract, has been used for in vitro studies of human reaginic allergy. The faithfulness with which this experimental system mimics certain aspects of human allergic disease, is borne out by the following recapitulation of our findings to-date.

a. Mechanistic studies of the histamine release phenomenon. The antigenic release of histamine from human leukocytes provides a highly sensitive index of an immune event upon a cell surface as well as a measure of clinical disease severity (11-13). The in vitro procedure conforms to the principles of immunologic specificity. Leukocytes which respond to ragweed pollen antigens, do not necessarily react with tetanus toxoid or vice versa (14). This and other information indicates that the cells may carry immunoglobulins of varying specificities. The considerations discussed in the preceding paragraph have encouraged the thought that this in vitro procedure, which surpasses the human skin test in terms of threshold antigen levels, might serve in studies of other human diseases.

The release of histamine is currently visualized as a specifically-induced enhancement of a normal physiological response. The releasing cell may not be injured as a result of this process which, indeed, requires a metabolically active cell for its completion. The evidence to support this assertion is drawn from the following observations.

1. Leukocytes exposed to 40° C or to the enzyme poison, diisopropylfluorophosphate, no longer support the antigenic release of histamine. Presumably this is due to the derangement of homeostatic mechanisms (11, 15).

2. The release of histamine requires divalent cations even when the reactions approach completion, suggesting that membrane damage does not occur during the extracellular translocation of this vasoactive amine.

3. As is well known, the interaction of specific antibody and complement injures the target cell membrane as shown by the enhanced efflux of intracellular potassium. This statement does not characterize the allergic reaction with peripheral leukocytes, a reaction in which histamine release may be observed in the absence of enhanced potassium loss (16).

2. Some practical applications of the leukocyte-histamine release procedure.

a. Assay of allergens. In a log dose response plot, the release of histamine is linearly related to the antigen concentration, in the response range of about 20-80%. This observation has been developed as a basis for the construction of an in vitro assay of the allergenic potencies of various pollen extracts. The method is simple, and provides greater reproducibility and sensitivity than the direct skin test (17).

b. Assay of blocking antibodies in human serum. A detailed study clarified the influence of normal and allergic human sera on the leukocyte-histamine release process (15, 18). Serum from nonallergic individuals may enhance the extent and rate of histamine release in a reaction limited by antigen. With adequate antigen levels, serum exerts little observable effect. The enhancing activity of nonallergic serum has not been associated with the complement system.

In contrast to the potentiation achieved with normal serum, serum from allergic donors reacts with ragweed pollen antigen in the fluid phase and thereby diminishes its efficacy as a histamine releasing agent from antibody-bearing leukocytes. This antigen-neutralizing capacity (ANC) of sera from allergic donors can probably be used as an *in vitro* assay for estimating the protective action of the "blocking" antibody in human allergy. This antibody has been observed in the serum of more than several hundred allergic donors tested thus far, whether or not they received desensitization therapy.

From an immunologic standpoint, the ANC obeys the kinetics of virus neutralization, in that a given amount of serum will bind a fixed percentage, rather than a fixed quantity of specific antigen. Since infinitely small amounts of antigen can trigger the anaphylactic reaction, a logical consequence of this antigen-neutralizing activity is that only very high levels of ANC can be expected to suppress the allergic response in any meaningful way (13).

c. *Combined clinical-laboratory studies of human pollinosis*. (In collaboration with Drs. Lawrence M. Lichtenstein, Philip S. Norman and Walter W. Winkenwerder). The first year of a carefully controlled clinical and laboratory study of the value of therapeutic ragweed pollen immunization in humans has been completed. A summary report has been published (13). One of the pertinent findings has been that the amount of ragweed pollen antigen required to release 50% of the histamine from a donor's cell, provides a reliable index of disease and symptom severity during the ragweed pollen season. The degree of cell sensitivity undergoes but slight change as a consequence of specific desensitization therapy, or environmental exposure to the ragweed pollen during the late summer or early fall seasons. In contrast, the level of antibody activity as judged by ANC increases markedly following injection of a highly purified antigen in microgram quantities (4 to 62 μg per patient per year). Sera of patients injected with placebo preparations showed no such change. These patients have been studied during the following year since it has been reported that clinically demonstrable alleviation of symptoms may not be manifest until after several years of therapy. This observation is entirely consistent with our findings concerning the mode of antigen neutralization by blocking antibody and the fact that minute quantities of residual unbound antigen can initiate the reaction sequence which terminates in the liberation of histamine. The results obtained in the second year of the study were entirely in accord with the earlier observations.

3. *In Vitro* Passive Sensitization of Human Leukocytes. (With Dr. David A. Levy)

Peripheral leukocytes from nonallergic donors can be rendered responsive to ragweed pollen antigen by prior incubation with serum from ragweed-sensitive donors. Presumably, it is the reaginic antibody which is fixed to the cell surface during this process. This portion of the laboratory's effort is of interest in three respects.

a. This reaction system provides a valid *in vitro* procedure for studies of immunoglobulin fixation to human cells, one of the early and key reaction steps in immunopathologic processes.

b. This procedure should facilitate characterization and quantitative estimation of human reaginic antibodies.

c. It may also serve as an *in vitro* substitute for the Prausnitz-Küstner reaction in clinical studies of human allergic disease.

Some of the experimental conditions which govern the *in vitro* sensitizing process have been established (19-22). The response to antigen is increased when sensitization is carried out at:

1. The pH optimum of 7.4.
2. 37° C.
3. Optimal dilution of serum (a donor variable).
4. Appropriate donor leukocytes—only about 20% of all cell donors provide cells which are capable of releasing histamine after incubation with serum and subsequent antigen addition.
5. Concentrations of EDTA approximating 0.004 *M*.
6. Concentrations of heparin approximating 1-10 μg per ml.

d. *Seasonal variation in leukocyte sensitizing activity*. The leukocyte sensitization assay has been used to study the natural history of reagin formation, and the effect of specific desensitization therapy. Serum specimens taken from untreated ragweed hay fever patients at various seasons were assayed for leukocyte-sensitizing antibodies (PLS_{50}). In most of these patients, the PLS_{50} titers were highest in the period immediately following ragweed pollination, i.e., Sept. 15 to about Nov. 15. The serum reaginic activities seemed to fall thereafter, reaching their lowest values in the summer months. The mean ratio of postseason to preseason titers for the sera of 12 patients tested over a three-year period was 3.1 with a range of 0.9 to 9.6.

Interestingly, the mean ratio for eight-patient years for individuals under treatment was 1.6, with a range of 0.7 to 2.5. These data point to a two-fold effect of therapeutic immunization. The serum blocking activity is increased, thereby rendering ineffectual much of the allergenic activity of ragweed pollen antigens. In addition, and possibly of greater importance, the activity and/or level of the disease-producing immunoglobulins is diminished.

C. The Allergic Response of Rabbit Platelets and Leukocytes. (With Drs. Reuben P. Siraganian, Antonio G. Secchi and Benedito Oliveira)

1. Platelets.

Several years ago we reported that the prior fixation of rabbit antibody does not constitute an essential reaction step in the allergic response of rabbit platelets (23). This observation suggested that the mechanism of histamine release from rabbit platelets differed substantially from that of human leukocytes. In the latter case, the mediating, homologous immunoglobulins are bound to the reactive cell. This fixation process occurs *in vivo*, or can be duplicated by appropriate *in vitro* procedures. The human leukocyte also differs from the rabbit platelet in that the former can discharge its store of vasoactive compounds in the absence of added serum factors. Moreover, different classes of immunoglobulins may be involved in the allergic response of the two cell types. These considerations, in addition to the ease of obtaining adequate supplies of a homogeneous cell type, provided the incentive for renewing our studies of the release of histamine from rabbit platelets by an unrelated immune reaction (24-26).

The mechanistic differences summarized above, and particularly the plasma requirement, suggested that the allergic response of rabbit platelets might be associated with alterations in membrane function. Estimation of potassium efflux rates from the platelets was therefore selected as a sensitive means for detecting changes in membrane activity. To implement these studies, the reaction system we described several years ago was modified slightly, to allow for a period of equilibration between the platelet-rich plasma and the radioisotope Rb^{86} (a convenient substitute for K^{42}). The cells were then freed of extracellular isotope and serum constituents by several cycles of centrifugation and resuspension in Tyrode's buffer. The reaction mixtures generally contained about 10^7 platelets per ml in 10% heparinized autologous plasma. When necessary, EDTA was added to a final concentration of 4×10^{-4} M to chelate the divalent ions contributed by the cells, plasma, diluent, etc. Calcium and magnesium were included as required. The allergic reaction was initiated when 0.1 μg ovalbumin N was added to the reaction mixture containing 4.0 μg rabbit-anti-ovalbumin N per ml.

In time course experiments the reaction was terminated by rapid chilling to 4° C and centrifugation in the cold. The supernatant fluids were then analyzed for their content of extracellular Rb^{86} and histamine. The extent of Rb^{86} efflux is expressed as the fraction of residual or intracellular content of the isotope. Estimates of the duration of the latent period and initial velocities for Rb^{86} efflux were obtained from semilogarithmic plots.

In the absence of an immune event, the rate of Rb^{86} efflux seems to vary with the physiologic state of the platelets and approximates 11.8 ± 1.04 x 10^{-3} min^{-1}. These values define the extent of normal membrane leakiness of the platelet to this cation. Under the impact of an immune reaction, the rates increase markedly, and have attained levels of 11.3 x 10^{-2} min^{-1}. As the rate increases, there is a corresponding shortening of the latent period which intervenes between the addition of antigen and the observed acceleration. Both histamine release and the rate of Rb^{86} efflux vary with a number of experimental conditions, i.e., reaction volume and temperature, plasma level, pH, divalent cation concentrations, and the ratio and quantity of the immune reactants. It was of interest to note that the latent period for an increased Rb^{86} efflux was always shorter than that required for the release of histamine, despite variations in the former, ranging from 1 to 25 min. Furthermore, all alterations in the experimental conditions affected both Rb^{86} efflux rates and histamine release in parallel fashion.

The cation requirements for these studies were established in the presence of EDTA which proved useful in differentiating the roles of Ca^{2+} and of Mg^{2+}. As is well known, EDTA binds Ca^{2+} preferentially with respect to Mg^{2+} (Ka for Ca^{2+} = 10.59, and for Mg^{2+} = 8.69). It was therefore possible to design experiments in which the level of free Mg^{2+} could be varied independently of Ca^{2+} concentrations, which ranged from 0.0 to 30 x 10^{-4} $\underline{M}$. This was achieved by the incorporation of EDTA in levels which were equimolar with the total cation contribution of the plasma and other constituents of the reaction mixtures. The estimated free Mg^{2+} level is 1.0 x 10^{-4} $\underline{M}$ for reaction mixtures containing 10% plasma. Experiments were also performed with constant levels of Mg^{2+} and varying amounts of Ca^{2+}.

The results of these experiments indicate that the allergic response of platelets with respect to enhanced Rb^{86} efflux and histamine release has a single divalent cation requirement, namely, Mg^{2+}. The optimal level of this metal has been found to vary within the range 4 ± 1 x 10^{-4} $\underline{M}$. When Mg^{2+} is absent, or present at suboptimal concentrations, Ca^{2+} can be substituted. However, when Mg^{2+} levels are maintained at about 5 x 10^{-4} $\underline{M}$, Ca^{2+} serves only to lengthen the lag phase and decrease the initial velocity for both the Rb^{86} and histamine responses.

Once these conditions were established it was possible to define some of the other parameters governing the response of platelets to an allergic reaction in a reproducible manner. As mentioned above, the rates of Rb^{86} efflux and histamine release vary directly with antibody concentration. At any antibody level maximal effects are observed at antibody:antigen weight ratios in the antibody excess zone, corresponding to twice those for the equivalence zone as described by quantitative precipitin analyses. Complexes in antigen excess are devoid of activity, and those at specified antibody excess ratios are maximally effective. These observations suggest that a unique configuration of immunoglobulins, induced by union with antigen, is required to activate the sequence of reactions leading to the increased efflux of Rb^{86} and the release of histamine.

2. Leukocytes.

Following the recent observations of Zvaifler and Becker on passive cutaneous anaphylaxis in the rabbit, we have ascertained that leukocytes from immunized rabbits carry immunoglobulins which mediate the release of histamine on interaction with antigen. Reactive leukocytes are found in many, but not all, rabbits for more than a month following a single injection of antigen. As in the human situation, the rabbit cells respond to antigen with the release of histamine, in the absence of added plasma or serum to the reaction mixture. The nature of the mediating immunoglobulin, its mechanism of action, and its relationship to the one described by Zvaifler and Becker remain to be explored.

D. Antibody Weight Analyses in Terms of Antigen-Binding Capacity.

(With Dr. Eugene Rodriguez)

We have recently completed the development of a method called mABC for estimating antibody weight in terms of antigen-binding capacity (27, 28). One of the important current problems in immunology relates to the nature of the immunoglobulins which participate in biological reactions, such as anaphylaxis. Recently, considerable data has been forthcoming to suggest the presence of an immunoglobulin endowed with specific anaphylactogenic properties. A serious difficulty encountered by many studies of this type is that the biological activities of the immunoglobulins are measured in terms of end point dilution titers. On this basis, it is difficult to compare different immunoglobulins unless there are gross qualitative differences. Comparisons on a specific weight basis would seem preferable, if possible.

In an attempt to resolve this problem, we have devised a method for an absolute estimation of the antibody-combining property in serum. The method involves a relatively simple modification, mABC, of the procedure described by Dr. Richard Farr several years ago. In brief, low levels of antibody in the range of 1-10 μg nitrogen are treated with increasing amounts of antigen labeled with I^{125}. The antigen is present in large excess, e.g., 200-500 μg of nitrogen. Under these conditions, it has been assumed that complexes are formed which have the molecular composition Ag_2Ab. Following the union of antigen with antibody under these conditions, in which there is no precipitation, all the globulins are precipitated at half-saturated ammonium sulfate, as described by Dr. Farr. Antigen in firm linkage to antibody is also precipitated and can be measured as the only radioactive constituent in the precipitate. In these reaction mixtures, where huge antigen excess conditions prevail, a plateau is reached since further addition of antigen does not increase the amount of precipitated radio-labeled HSA. This is taken to mean that the complex is in the form Ag_2Ab. The advantages of this technique are apparent since it involves no prior treatment of the serum, nor any selection of a specific class of antibody. Initial studies suggest that the antibody formed in rabbits following a single toe-pad injection is primarily 7S rather than 19S, when estimated on a weight basis. This conclusion emerges from combined hemagglutination and mABC studies of eluates from Sephadex G-200 columns. It has been found that almost all the hemagglutinating antibody is in the 19S fraction but that the molar ratios of 7S to 19S antibody may exceed 20:1. In addition to the preponderance of 7S immunoglobulins, use of the mABC procedure divulged the presence of nonprecipitating or coprecipitating antibodies. These became manifest in the sera of rabbits yielding higher mABC antibody weight estimates than were obtained from precipitin analyses. This observation, in turn, suggests a practical method for obtaining nonprecipitating antibody free of those immunoglobulins capable of forming insoluble aggregates with antigen.

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13. ABSTRACT

The Progress Report which follows summarizes two studies. The first deals with experimental infection of the rat with Plasmodium berghei. These studies include the use of an electronic particle counter to estimate the degree of parasitemia in the infected rat, and the development of an assay procedure for the protective action of humoral antibody. Indirect evidence has also been obtained which points to the merozoite or trophozite as the developmental forms which react with the antibody that suppresses or prevents infection with P. berghei.

The second investigation is a continuation of this laboratory's interest in mechanisms of hypersensitivity. In vitro studies with rabbit platelets and rabbit leukocytes point to the existence of two different classes of immunoglobulins capable of mediating the release of vasoactive compounds. One immunoglobulin which is reactive with the platelet is present in hyperimmune sera. It does not bind to the platelet as a prelude to interaction with antigen. The immunoglobulin which releases histamine from washed suspensions of rabbit leukocytes is bound to these cells and appears during the early stages of immunization. It may be the same immunoglobulin which mediates PCA in the rabbit, as described by Becker and his colleagues. The platelet and leukocyte reaction systems also differ in their requirement for plasma. The plasma requirement is only demonstrable in the allergic response of rabbit platelets. Rabbit leukocytes respond to antigen in the absence of plasma in the fluid phase.

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Terminal Report

Studies on the Mode of Action of Diphtheria Toxin

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A. M. Pappenheimer, Jr., Harvard University

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Terminal report to the Commission on Immunization of the Armed Forces Epidemiological Board

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ABSTRACT

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Principal investigator: A. M. Pappenheimer, Jr.

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Annual Report to the Commission on Immunization of the Armed Forces Epidemiological Board

Using the technique of radioautography, it has been demonstrated that a maximum of 25-50 molecules of carrier-free iodine-125-labeled diphtheria toxin is bound per cell by human HeLa cells treated with approximately 10^7 labeled toxin molecules per cell. Radioautographs of sections of labeled cells locate most if not all of the toxin fixed in the cytoplasmic membrane. Under identical conditions, toxin-resistant mouse L cells fix very much less label. It has now been demonstrated that diphtheria toxin is a powerful NADase of a hitherto unknown type. Both *in vitro* and *in vivo*, toxin catalyses the release of free nicotinamide from NAD with the simultaneous transfer of its ADP-ribose moiety to a point at or near the active site of aminoacyl-transferase II. The result is inhibition of protein synthesis. Transferase II (non-bacterial) appears to be the unique protein acceptor for the ADP-ribose moiety. NADases from other sources (bacteria, Neurospora, animal tissues) do not inactivate transferase II. The reaction can be reversed in intoxicated extracts, whether from normal or from intoxicated cells, provided *both* toxin and an excess of nicotinamide are present. The mechanism in described by the following equations

1. $\text{Tx} + \text{NAD} \rightleftharpoons \text{Tx-NAD}$
2. $\text{Tx - NAD} + \text{Tfase II} \rightleftharpoons [\text{Tx - NAD - Tfase II}]$
3. $[\text{Tx-NAD-Tfase}] \rightleftharpoons \text{ADPR-Tfase} + \text{Tx} + \text{nicotinamide}$

Sum: $\text{NAD} + \text{Tfase} \underset{\text{(toxin)}}{\rightleftharpoons} \text{Nicotinamide} + \text{ADPR - Tfase}$

For the past 10 years, the Commission on Immunization has given its support to our Studies on the Mode of Action of Diphtheria Toxin. It is most gratifying to me that in my terminal report to the Commission, we are able to provide a final answer to the problem originally posed. At least in the case of mammalian cells growing in tissue culture, we are now able to explain the mode of action of diphtheria toxin in molecular terms. It has been demonstrated that a maximum of 25-50 toxin molecules, located in the cell membrane, catalyse the hydrolysis of nicotinamide adenine dinucleotide (NAD) with liberation of free nicotinamide and transfer of the ADP-ribose moiety to a point at or near the active site of the essential enzyme--aminoacyl-transferase II. This enzyme, acting on the ribosomes, is required for transfer of all amino acids from aminoacyl-tRNA to the growing peptide chain (1,2). It is probably a "translocase" that catalyses the movement of the growing chain on the ribosome so as to be in position to accept the next amino acid from mRNA (3). When a saturating dose of toxin is added to a culture of HeLa cells, a few toxin molecules bound to the cell membrane suffice to catalyse the conversion of the entire cell content of free transferase II to its inactive ADP-ribosyl derivative within a few minutes. As a result, soon afterwards protein synthesis and cell growth come to a complete standstill.

Interaction of toxin with NDA: In my previous report (see also 4,5,6), I presented evidence for a mechanism of toxin action *in vitro*, first involving the formation of a dissociable NAD-toxin complex, followed by the further stochiometric interaction with transferase II to form a relatively undissociable, enzymatically inactive, Tx-NAD-Tfase II complex. By means of equilibrium dialysis we have shown that each mole of diphtheria toxin reversibly binds one mole of NAD with an association constant of about 2.3×10^3 liters/mole. This interaction was demonstrated independently by Montanaro and Sperti (7) who also showed, using the technique of fluorescence quenching, that the toxin binds NADH and NADPH some 100-1000 times more strongly than NAD itself. The interaction is also being studied in Dr. N. Kaplan's laboratory at Brandeis University where they have recently been able to obtain direct evidence in the ultracentrifuge for a Tx-NAD-Tfase II complex. In our laboratory we have been able to demonstrate binding of nicotinamide and of adenosine by toxin (see also Sperti and Montanaro, 8) and with Mr. John Philbrick we have shown that NAD, nicotinamide and NADH all compete for the same site on the toxin molecule.

Quantitative relationships: Using the above model, we were able to derive the following equation based on the Mass Law which accurately predicts the percent inhibition of amino acid incorporation in cell free systems at any given toxin and NAD concentration (6) provided both are in excess:

$$\% \text{ inhibition} = \frac{100\ [Tx]\ [NAD]\ K_1K_2}{1 + [Tx]\ [NAD]\ K_1K_2}$$

where $K_1 = 2.3 \times 10^3$ L/mole is the association constant for the interaction of toxin and NAD, and $K_2 = 1.4 \times 10^{10}$ L/mole is a constant that determines the affinity of the Tx-NAD complex for transferase II. According to this equation there should be a reciprocal relationship between [Tx] and [NAD]. In figure 1, we have plotted the concentrations of Tx and NAD required for 50% inhibition of aminoacyl transfer using a polyU-directed ^{14}C-phenylalanine incorporating system from rabbit reticulocytes (see 9). It will be noted that the product $[Tx] \times [NAD] = K_1K_2 = 4 \times 10^{-13}$ remains constant over at least a 1000-fold range of concentrations. As the figure shows, deviation from linearity only occurs at very low toxin concentrations. At low toxin concentration, the rate of transferase II inactivation becomes slow enough to measure. In figure 2, the effect of temperature on the toxin-mediated inactivation of transferase II is compared, before and after preincubation, with the effect of temperature on the spontaneous decay of this unstable enzyme. The Q10 for the inactivation is 2.1, a value characteristic of most enzymatic reactions.

Site of toxin action in living cells (10): It may easily be calculated that a "saturating dose" of toxin (concentration required to arrest protein synthesis in HeLa cell cultures in a minimum length of time) contains toxin in a concentration (ca. 4×10^{-8}M) approximately equivalent to the aminoacyl-transferase content of the cells or at least to their ribosome content. However, we know from our previous studies and those of others, that toxin concentrations several orders of magnitude lower than this, arrest protein synthesis and growth after an extended latent period. In view of the apparent discrepancy between the concentration of toxin required to inhibit growth of HeLa cells *in vivo* and that required to inhibit aminoacyl transfer activity *in vitro*, it seemed important to determine as precisely as possible the maximum number of toxin molecules fixed per cell in the presence of a saturating dose. This was accomplished with HeLa cells using highly purified toxin, trace-labeled with carrier-free iodine-125 (2 atoms iodine per molecule toxin), and the technique of radioautography (10). The results (see Table 1) showed that a maximum of only 25-50 molecules of labeled toxin were bound per cell by HeLa cells treated for several hours with about 10^7 molecules per cell. Radioautographs of sections of labeled cells have located the toxin molecules bound in the peripheral cell membrane. Under identical conditions, mouse "L" cells bind little or no toxin (even though extracts from them are sensitive,(6,11)). Failure of mouse cells to bind the toxin provides a satisfactory explanation for the well known resistance of this species to diphtheria toxin. There is no binding of toxin to HeLa cells in the presence of antitoxin. Since antitoxin does *not* inhibit the action of toxin on the aminoacyl transfer system extracted from HeLa cells, it is clear that its protective action *in vivo* is due to the fact that toxin cannot reach the cell surface after combination with specific antibody. Once toxin is bound to the HeLa cell membrane it can no longer interact with agents added from without such as specific antitoxin or trypsin (10). Nevertheless, membrane-bound toxin must be in contact with cytoplasmic components, since even this small number of toxin molecules suffices to reduce the intracellular transferase II to a very low level within a few minutes. Since we suspect that there must be at least as many transferase II molecules in each HeLa cell as there are ribosomes, the number of which has been estimated to be about 10^7, each toxin molecule must cause the inactivation of more than 10^5 molecules of transferase II per minute in the living cell. Can we account for such a high turnover number *in vivo* on the basis of the inhibition of aminoacyl transfer by toxin observed in cell extracts?

Since the volume of 2×10^3 packed HeLa cells is about one ml., it follows that the maximum cell-bound concentration of toxin will be 1 to 2×10^{-11}M. Taking the intracellular concentration of NAD to be 5×10^{-4}M (6), then the product [NAD] x [toxin] for intoxicated cells must lie between 0.5 and $1 \times 10^{-14}M^2$. From our published date (6), we know that the transferase II activity of HeLa extracts is 50% inhibited when the [NAD] x [toxin] product is about $3 \times 10^{-14}M^2$, irrespective of the precise toxin concentration. We feel that the agreement between these two figures is quite remarkable, particularly in view of the several assumptions that have been made. Thus we do not know what fraction of the intracellular NAD is available for interaction with the toxin; nor do we know how best to compare reaction rates *in vitro* with those observed *in vivo*. In any event, it does not seem necessary at this time, to postulate the enhancement of toxin activity *in vivo* by some unknown membrane component.

Toxin-stimulated hydrolysis of NAD (11,12): The calculations of the preceding section suggest that the same mechanism of intoxication is operating in living cells as in aminoacyl transfer systems extracted from them. It is obvious, however, that the previously suggested simple stochiometric interaction between free transferase II and a toxin-NAD complex (6) fails to provide an adequate explanation for the rapid inactivation of the incorporating system by toxin acting on living cells. Moreover, despite repeated efforts, we have been unable to obtain any direct evidence for the postulated, stable NAD-containing toxin-transferase complex using ^{125}I-labeled toxin and ^{14}C-labeled NAD. We therefore decided to investigate the possibility that toxin was causing the splitting of the NAD molecule in the presence of the aminoacyl transfer system.

That toxin, concomittant with the inactivation of transferase II does indeed stimulate hydrolysis of NAD with release of free nicotinamide is shown in figure 3. In these experiments, a high concentration of toxin was allowed to react with soluble enzymes (AS65 fraction) from reticulocytes in the presence of NAD labeled with ^{14}C in the carbonyl position. After incubation at 37C for 10 minutes, the mixture was deproteinized and the supernate filtered through a Sephadex G10 column. Figure 3a shows that when toxin and ^{14}C (carbonyl)-NAD were incubated in the absence of AS65, only about 5% (already present due to spontaneous breakdown of NAD) of the label eluted with the nicotinamide fraction. Figure 3b shows a small increase in nicotinamide when NAD was incubated with AS65 in the absence of toxin due to a trace of NADase present in the AS65 fraction. When both toxin and the aminoacyl-transferase-containing AS65 fraction were present, most of the NAD was hydrolysed and 90-95% of the label appeared in the nicotinamide fraction. These results suggest that in the presence of transferase II, diphtheria toxin stimulates the hydrolysis of NAD with release of free nicotinamide under the identical conditions leading to inactivation of the aminoacyl transfer system.

Reversibility of inactivation: The reversibility of the intoxication reaction *in vitro* was first shown by Goor *et al.* (6), who found that aminoacyl transfer activity could be restored by addition of relatively high concentrations of nicotinamide. Since toxin was already present in the system, its importance for the reversal was not recognized. Table 2 shows that nicotinamide is far less effective in reactivating the aminoacyl-transferase activity in an intoxicated reticulocyte AS65 preparation when the toxin is first removed by specific precipitation with diphtheria antitoxin. Appreciable transferase activity is only restored to the system by adding back free toxin or the toxin-antitoxin floccules themselves. The small stimulation of incorporation by nicotinamide alone is doubtless due to catalysis by a trace of soluble toxin-antitoxin complex that failed to precipitate within 20 minutes at 25C.

When it was first shown that nicotinamide could reverse the action of toxin in cell-free extracts by competition with NAD, we attempted unsuccessfully to reactivate extracts prepared from intoxicated HeLa cells by adding nicotinamide to them. Table 3 shows that both nicotinamide and toxin are required for restoration of aminoacyl transfer activity in extracts from intoxicated cells; in other words, one must add back the very agent that intoxicated them in the first place.

Our finding that the toxin-stimulated release of nicotinamide from NAD is closely linked to inhibition of transferase II and that the inactivated enzyme can be reactivated again, provided both toxin and nicotinamide are present, can best be explained by assuming that toxin catalyzes the reversible transfer of ADP-ribose from NAD to transferase II. The reaction probably involves the formation of an unstable toxin-NAD-enzyme intermediate according to the following scheme:

$$\begin{array}{ll} 1. & \text{Tx} + \text{NAD} \rightleftharpoons \text{Tx-NAD} \\ 2. & \text{Tx-NAD} + \text{Tfase II} \rightleftharpoons [\text{NAD-Tfase-Tx}] \\ 3. & [\text{NAD-Tfase-Tx}] \rightleftharpoons \text{ADPR-Tfase II} + \text{Tx} + \text{nicotinamide} \\ \hline \text{Sum} & \text{NAD} + \text{Tfase II} \rightleftharpoons \text{ADPR - Tfase II} + \text{nicotinamide} \end{array}$$

It was at this point that we received from Dr. I. Kato of Tokyo, the manuscript of a paper by Honjo et al (12) showing that diphtheria toxin catalyzes the reversible transfer of the ADP-ribose moiety from NAD to a point at or near the active site of transferase II purified from rat liver. Using NAD labeled in the adenine ring and ^{32}P-labeled NAD, the Japanese workers have shown that the ADP-ribose is linked covalently to the enzyme through its ribose-5' phosphate end since treatment with snake venom phosphodiesterase liberates AMP leaving ribose phosphate attached to the toxin.

In conclusion then, our studies have shown that a maximum of 25-50 molecules of diphtheria toxin, bound to the cell membranes of HeLa cells, arrest growth and protein synthesis by catalysing the release of free nicotinamide from NAD and conversion of the entire intracellular content of free transferase II to its inactive ADP-ribosyl derivative. In cultures of living cells, intoxication has so far proved to be irreversible. However, extracts from intoxicated cells can be restored to full aminoacyl transfer activity by addition of small amounts of toxin together with excess nicotinamide.

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Figure 1: Toxin and NAD concentrations required for 50% inhibition of aminoacyl transfer.

Each point on the graph was obtained by a separate set of measurements. For each set a number of 5 µl aliquots of reticulocyte AS65 fraction were incubated for 10 minutes at 37°C with a fixed amount of NAD and differeent amounts of toxin. The aminoacyl transfer activity remaining was then assayed in the usual manner. The results were plotted against toxin concentration and from this curve was read the amount of toxin necessary to reduce the number of counts incorporated by 50%.

Figure 2: Effect of temperature on inactivation of reticulocyte transferase by toxin.

Tubes with complete reaction mixtures containing reticulocyte AS65 fraction and lacking only ^{14}C-Phe-tRNA, were incubated with 20 µM NAD for 10 minutes at the temperatures indicated. The tubes were then quickly cooled to 0°C, ^{14}C-Phe-tRNA was added to each and the incubation continued for 40 minutes at 37°C.

Curve A - No toxin added

Curve B - preincubated with 0.75 Lf/ml toxin

Curve C - 0.75 Lf/ml toxin added after preincubation

Figure 3: Toxin-stimulated hydrolysis of NAD

^{14}C-(carbonyl) NAD was incubated at 37°C for 10 minutes in 2 ml standard assay buffer, either (a) with 3 x 10^{-6}M diphtheria toxin alone or (b) with 2 mg/ml reticulocyte AS65 fraction alone or (c,d) with both together. An equal volume of 10% TCA containing 10^{-4}M NAD and 10^{-4}M nicotinamide was added and the precipitate removed by centrifugation. The supernate was extracted 3 times with 8 ml changes of ether and the aqueous layers perevaporated in dialysis tubing to a volume of 0.5 ml. Each sample was then applied to a small Sephadex G10 column (V_o = 3.5 ml). Elution of NAD (I) and nicotinamide (II) was monitored at 260 mμ and 0.5 ml fractions were collected on glass fiber pads, dried and counted by liquid scintillation. The ^{14}C-NAD concentrations were 2 x 10^{-7}M in (a) and (b), 4 x 10^{-7}M in (c) and 8 x 10^{-4}M in (d).

TOXIN and NAD CONCENTRATION REQUIRED FOR
50% INHIBITION of AMINOACYL TRANSFER
(Poly U, ^{14}C-Phe-tRNA)

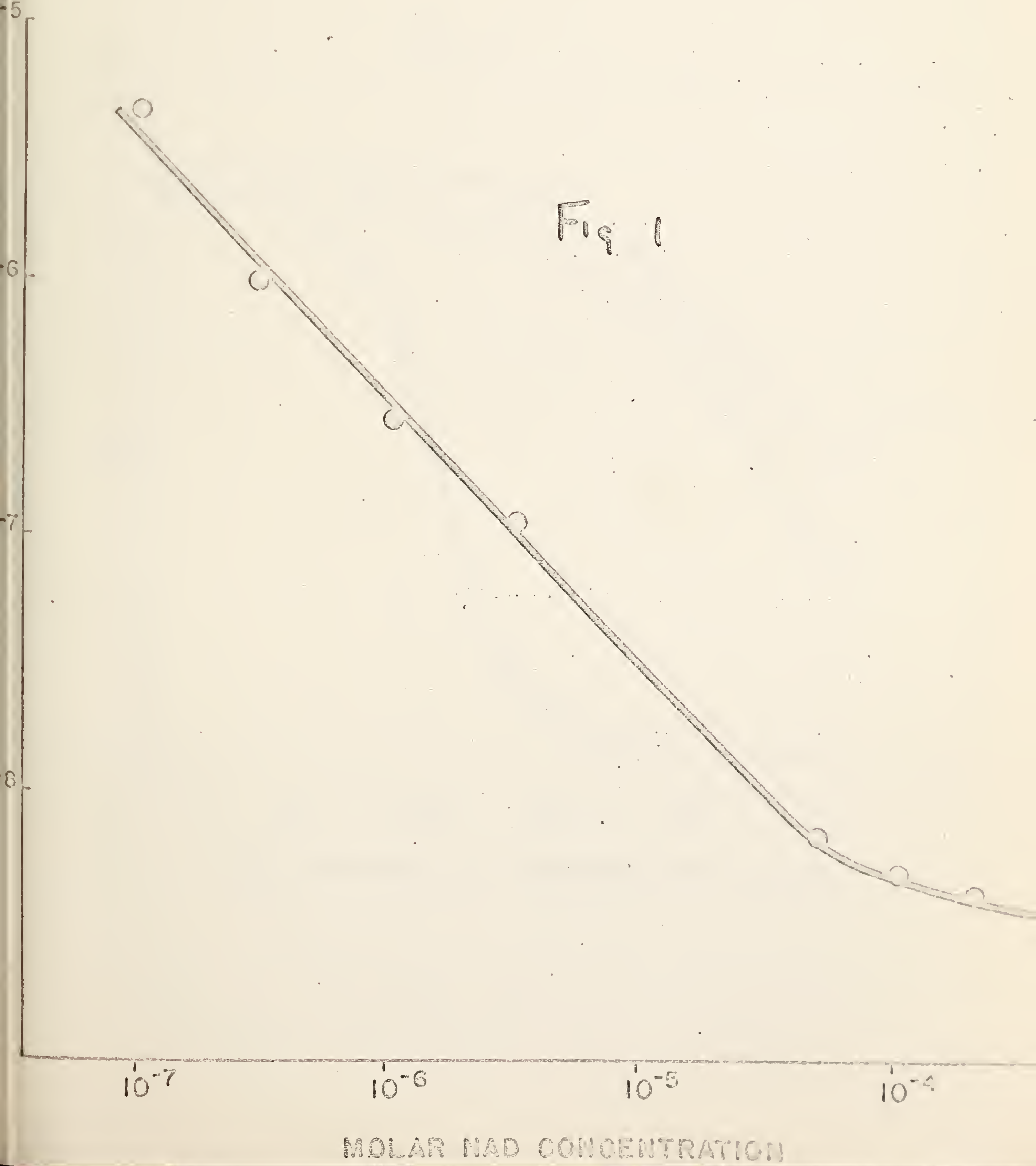
Fig 1

Fig 2

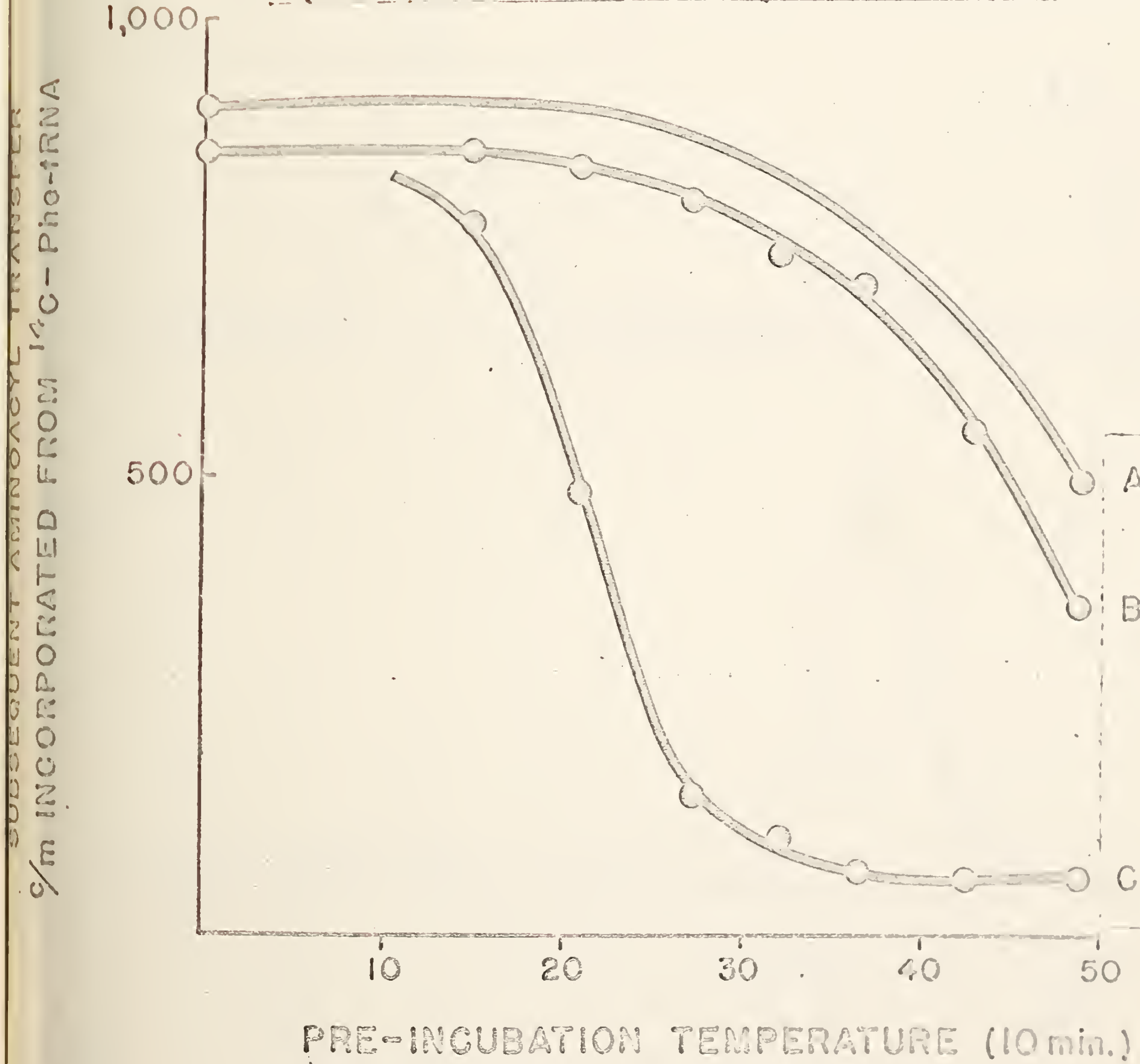
1,000
500
SUBSEQUENT AMINOACYL TRANSFER
c/m INCORPORATED FROM ^{14}C-Phe-tRNA
10
20
30
40
50
PRE-INCUBATION TEMPERATURE (10 min.)
A
B
C

Fig 3

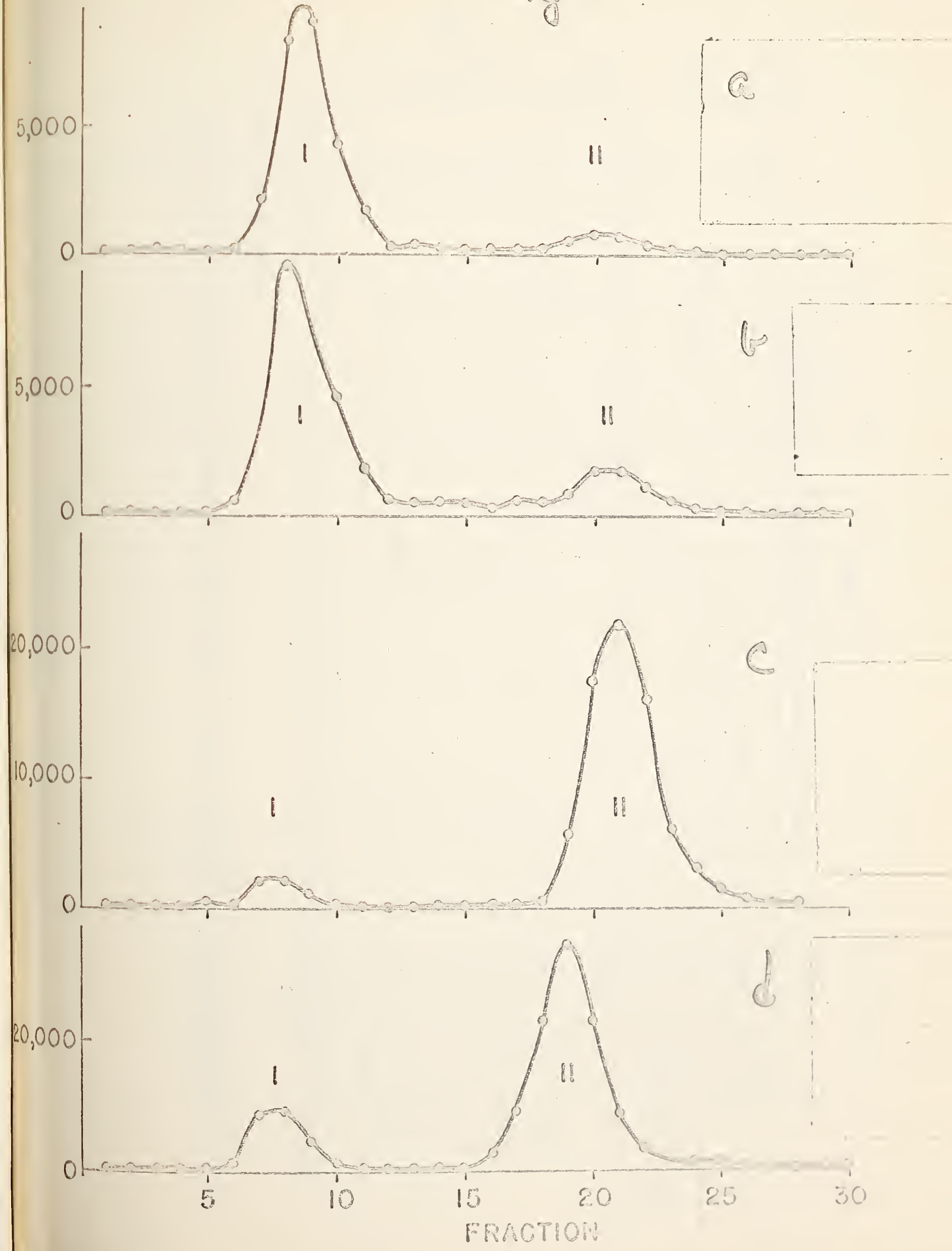
a
5,000
0
I
II
b
5,000
0
I
II
c
20,000
10,000
0
I
II
d
20,000
0
I
II
5
10
15
20
25
30
FRACTION

TABLE 1

FIXATION OF $^{125}I_2$-TOXIN BY MAMMALIAN CELLS*

Time	^{125}I decay	Average number of grain counts per cell‡		
		HeLa	Mouse "L"	HeLa + excess unlabeled toxin
days	%			
7	7.5	6.9	1.9	1.6
14	15	12.1	6.0	2.6
21	21	15.1	7.8	5.0
28	28	22.0	11	10
60	50	(40)§	(20)	(18)

*Cells treated 6 hr with 0.13 Lf/ml $^{125}I_2$-toxin at 37°C before washing and preparing for radioautography.

‡Each figure represents average of more than 100 cells corrected for background counts.

§Estimated counts in parentheses.

Table 2

Toxin Requirement for Reversal of *in vitro* Intoxication

Except for the control (A) from which toxin was omitted, 50 μl reticulocyte AS65 fraction per ml of standard assay buffer containing 0.5 μM NAD and 100 Lf/ml diphtheria toxin were incubated at 37°C for 10 minutes. An equal volume of antitoxin (100 units/ml) was then added. After a further 20 minutes at 25°C, the specific floccules were removed by centrifugation. Assay of the supernates for aminoacyl transfer activity, using 50 μl aliquots, was carried out in the presence of 5 mM nicotinamide except in the case of (B). In (C) there were no further additions; in (D) free toxin was added; in (E) the toxin-antitoxin floccules previously removed were added back and in (F) freshly prepared washed toxin-antitoxin floccules were added in the same concentration as free toxin.

Reaction Mixture	Aminoacyl Transfer Activity (cpm)
(A) Normal AS65 control	562
(B) Intoxicated AS65 supernate (toxin removed)	50
(C) Intoxicated supernate + nicotinamide (5 mM)	130
(D) Same as (C) + 50Lf/ml fresh toxin	539
(E) Same as (C) + Tx-antiTx floccules	423
(F) Same as (C) + fresh washed floccules	405

Table 3

Reactivation of Transferase II in AS70 Fraction

Prepared from Intoxicated HeLa Cells.

Additions	Aminoacyl transfer activity in AS70 preparation: From intoxicated cells	From normal cells
	cpm	cpm
None	103	568
toxin, 5 Lf/ml	88	536
nicotinamide (5mM)	106	565
toxin (5Lf/ml) + Nicotinamide (5mM)	612	577
toxin (5Lf/ml) + NAD (10μM)	78	142
No AS70 fraction	15	-

AS70 fractions were prepared from normal HeLa cells and from cells harvested from a parallel culture grown for 4 hours in the presence of 1 Lf/ml toxin. Amino acid incorporation stopped within 2 hours in the intoxicated culture. Aminoacyl transfer activity was assayed without preincubation.

AD__________

ANTIBODY FORMATION (U)

ANNUAL PROGRESS REPORT

by

Jonathan W. Uhr, M.D.

1 July 1968
(For the period 1 March 1967 to 30 June 1968)

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Office of The Surgeon General, Washington, D.C. 20315
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SUMMARY

Immunoglobulin Synthesis

The mechanisms underlying incorporation of carbohydrate into immunoglobulin were investigated. It was shown that glucosamine, the first sugar in the carbohydrate moiety, is added primarily onto nascent chains whereas mannose and galactose are incorporated primarily onto completed chains.

Antibody Regulation of Antibody Synthesis

Rabbits were passively immunized to bovine serum albumin (BSA) and bacteriophage øX. Their blood was allowed to flow through glass tubing containing an immunoabsorbent with covalently bound BSA. Within 2 hours, serum anti-BSA was reduced about fifty percent whereas anti-øX titers were unaffected.

Streptococcal Infections in Baboons

Local persistent skin and pharyngeal infections were induced by injection of virulent organisms accompanied by small amounts of croton oil. These infections were usually accompanied by a vigorous immune response and in one of a dozen animals by a severe "lymphoid" myocarditis.

Immunoglobulin Synthesis

Several major features of assembly of the light and heavy chains have been clarified and were summarized in an earlier Progress Report. During the last year, we have concentrated on the mode of incorporation of the carbohydrate moiety and on preparation for studies that will determine the organelles and intracellular sites in which particular biosynthetic events occur.

Thus, using plasma cell tumors of mice, it has been shown that glucosamine is incorporated into nascent chains and that the majority of incorporated label is releasable by *in vitro* treatment of polysomes with puromycin. In contrast, galactose and mannose appear to be added predominantly onto released polypeptide chains.

Endoplasmic reticulum from these same plasma cell tumors and also from rabbit lymph nodes has been separated into smooth and rough fractions by density gradient centrifugation (Jamieson, J.D., and Palade, G.E., *Proc. Nat. Acad. Sci.*, *55*, *424*, 1966). Electron-microscopic examination of these fractions has confirmed their relative purity. Thus, the rough fraction is composed entirely of ribosome-lined vesicles containing electron dense material which presumably is protein present in the cisternae of the cells before their disruption. Smooth endoplasmic reticulum appears to be free of membrane-bounded ribosomes and to contain pieces of the Golgi apparatus. We can therefore proceed with pulse-chase experiments as outlined in the research plan. At the same time that fractionation procedures were being developed for plasma cells, we attempted to obtain preliminary information on these problems by ultrastructure radioautography. These experiments were done in collaboration with Dr. Daniel Zagury of Centre Rene Huguenen in Paris and Dr. Palade and his coworkers at the Rockefeller University. Preliminary results indicate that sufficient isotope was incorporated in these pulse-chase experiments to allow quantitative study of grain counts in different parts of the cell. These studies will continue along with the biochemical approach.

Antibody Regulation of Antibody Synthesis

Serum antibody was specifically removed from passively immunized rabbits by the following procedure: bovine serum albumin (BSA) was covalently bound to cellulose to form a particulate immunoabsorbent. The immunoabsorbent is remarkably stable, e.g., resists dissociation in 5 M urea. After cannulation of the femoral artery and vein of a rabbit passively immunized to BSA and bacteriophage øX, blood was allowed to flow through the immunoabsorbent contained within a section of glass tubing for 2-6 hours. Aliquots of serum obtained from the femoral artery were used to determine antibody levels to BSA (Farr technique) and to bacteriophage øX (neutralization). Antibody levels to øX remained constant throughout the above procedure. In contrast, antibody levels to BSA fell to approximately fifty percent of the pre-cannulation level. The fall was usually completed by 2 hours after which the anti-BSA levels plateaued.

These results establish the feasibility of specific removal of antibody. Such an approach may be useful to study the regulatory effect of serum antibody in the immune response and is of potential clinical interest.

Streptococcal Infections and Immunity in Primates

We have performed pilot studies of streptococcal infections and the resulting antibody response in twelve baboons. These experiments were designed to determine the means by which persistent local streptococcal infections could be established in the throat or skin. Such infections in man are most likely to be associated with the sequelae of rheumatic fever and glomerulonephritis. Each animal was given 4-5 infections usually with strains of different types. The strains varied considerably in their virulence for baboons. Persistent skin and pharyngeal infections were obtained consistently by injecting with the streptococci small amounts of croton oil in order to induce simultaneous necrosis. Serial antibody titers were performed to streptolysin O in all animals and antibody titers to streptokinase and hyaluronidase were performed on smaller numbers of sera. Vigorous antibody responses were thus documented, with anti-streptolysin O titers often exceeding those observed in naturally occurring streptococcal infections of man.

Rechallenge in the skin with a streptococcal strain previously injected in the throat resulted in less persistent skin infections and no rise in ASLO. The question of whether superficial skin infections in the absence of throat infection can induce type specific immunity requires further study.

In regard to sequelae, histologic material is still in the process of evaluation. At least one animal showed severe myocarditis with focal muscle necrosis and focal infiltration with lymphoid-histiocytic cells. A number of experimental animals showed glomeruli which were larger and more cellular than those seen in control animals.

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ANTIBODY FORMATION (U)

ADDENDUM TO
ANNUAL PROGRESS REPORT

by

Jonathan W. Uhr, M.D.

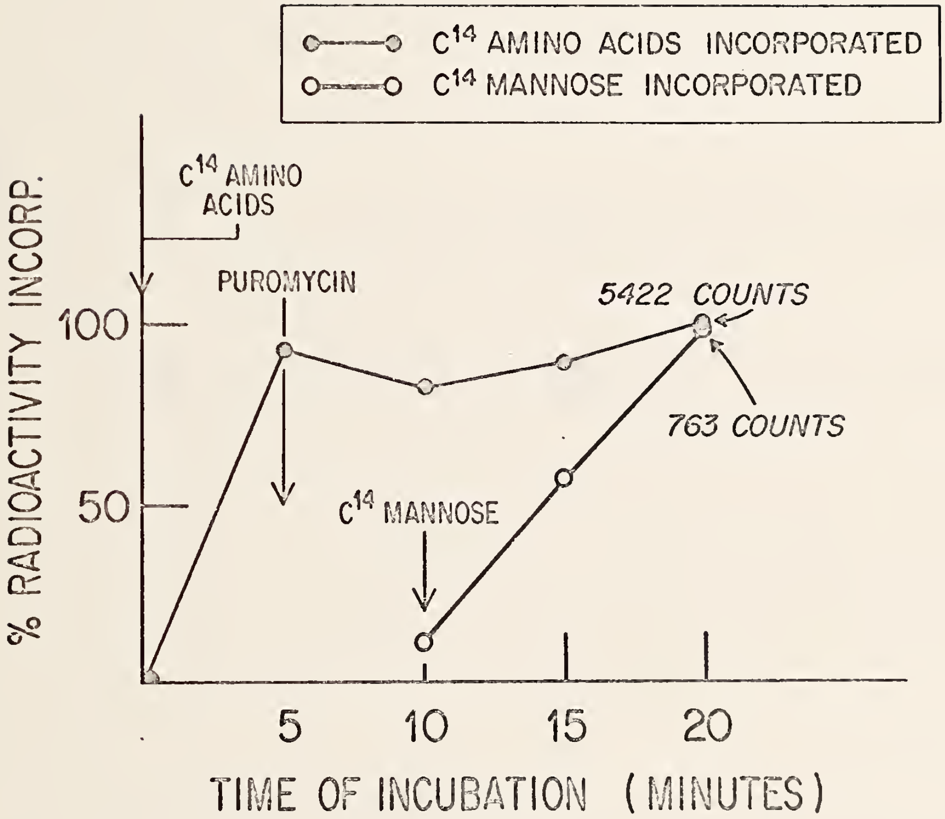
(For the period 1 March 1967 to 30 June 1968)

FIGURE 1

Sucrose gradient fraction	OD_{280}	Total cts/5 min	Cts/1.0 OD_{280}
Polysomes	2.283	6,000	2,628
Single ribosomes	0.897	1,025	1,142
Top	1.75	6,205	3,571

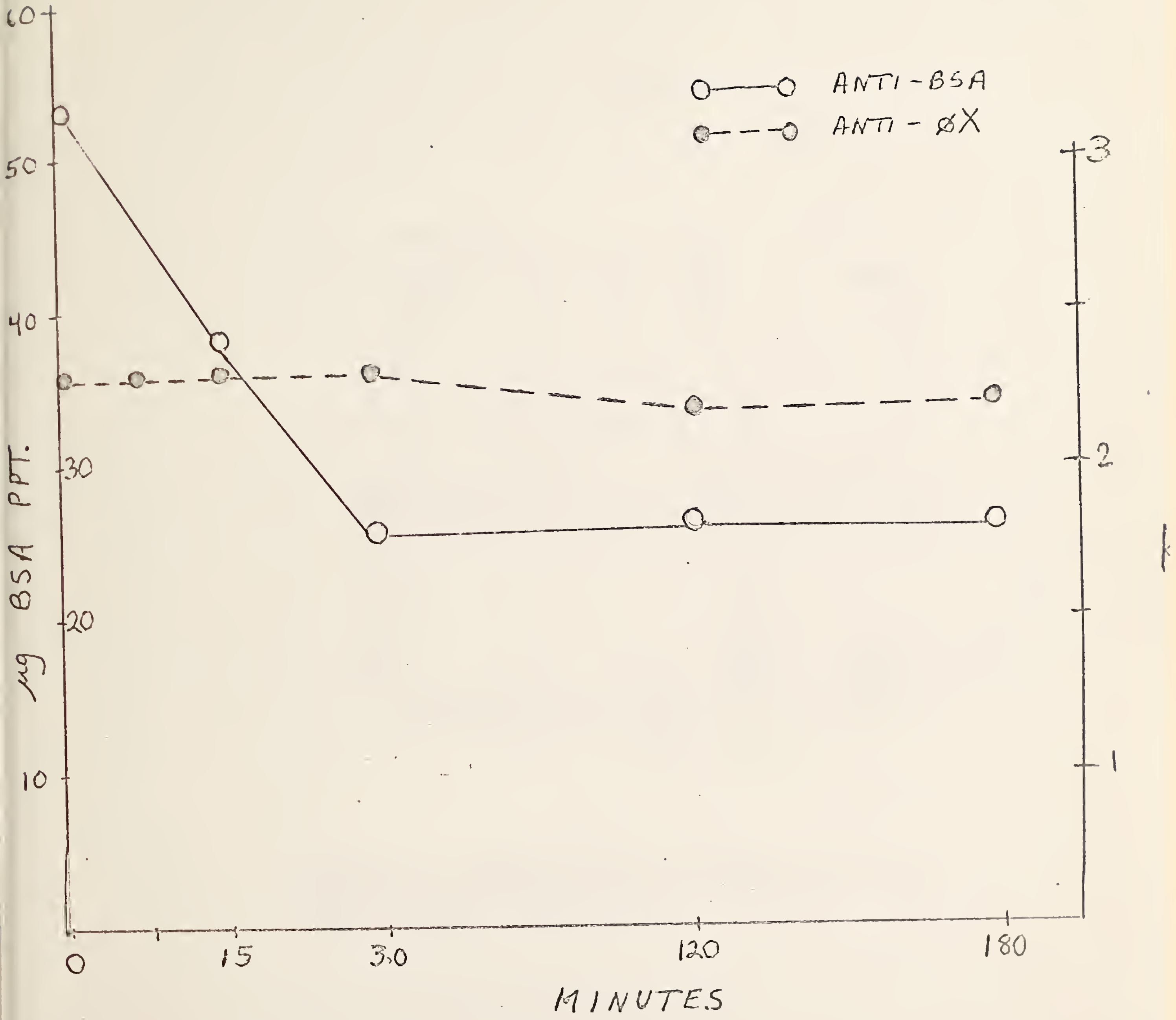
Incorporation of ^{14}C-glucosamine into nascent chains on polyribosomes. 2.5 x 10^8 MPC_{11} tumor cells were exposed to 25 μc of ^{14}C-glucosamine for 35 min. The cell lysate was separated on a 15-30⁰/o sucrose gradient and nascent chains were released from polysomes and single ribosomes by puromycin. The CPM/OD are shown for these puromycin-released chains as well as the completed chains which are present at the top of the gradient.

FIGURE 2



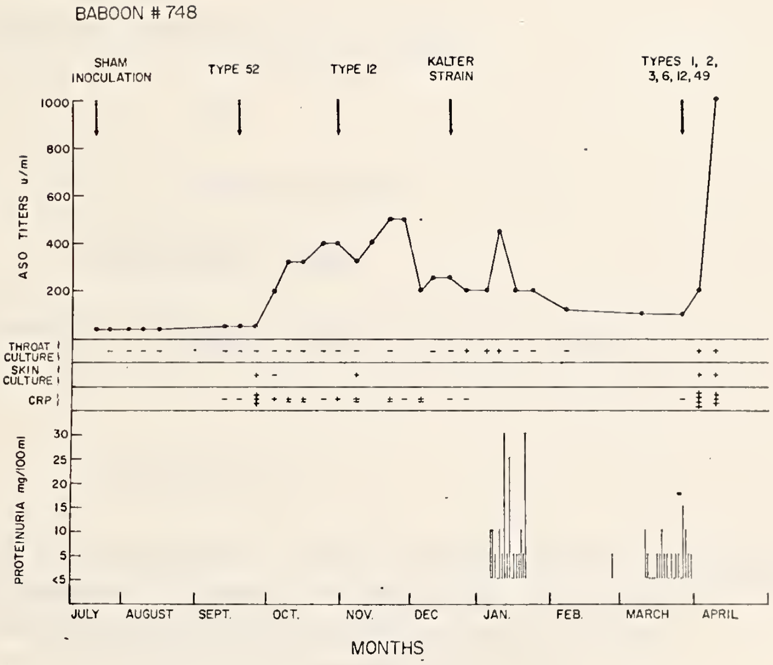
Incorporation of ^{14}C-amino acids and ^{14}C-mannose into TCA precipitable material by MPC_{11} cells. ●—● 5 x 10^7 cells were exposed to 10 µc of ^{14}C-amino acids for 5 min. and were then treated with 2 x 10^{-4} M puromycin. O—O 5 x 10^7 cells were treated with 2 x 10^{-4} M puromycin for 5 min. and were then exposed to 10µc of ^{14}C-mannose.

FIGURE 3



Removal of antibody by an immunoabsorbent. A 3-kg rabbit was provided with 16 mg rabbit anti-BSA and anti-ØX (1 ml of a K of 2700). An extracorporeal circulation was established in which the blood flowed through a chamber containing BSA bound to bromacetyl cellulose. Antibody determinations (Farr technique) and neutralization assays were performed at intervals. The extracorporeal circulation was initiated at time 5'.

FIGURE 4



Summary of response of one baboon to four streptococcal infections. On autopsy, there was acute glomerulonephritis.

AD ______________________

RESPONSES OF HUMANS TO CHOLERA VACCINE IMMUNIZATION (U)

ANNUAL PROGRESS REPORT

by

W. F. Verwey, Sc.D.

September, 1968

(For the period 1 February 1967 to 30 June 1968)

supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Office of the Surgeon General, Washington, D.C. 20315
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SUMMARY

Volunteers in a unit of the Texas Department of Corrections have been employed in the study of serological responses to cholera vaccines administered by various regimes. The three vaccines used were 1) the one found to be effective in the Pakistan field trials of 1963 and 1964 (CRL), 2) a commerical vaccine of normal mouse-protective potency (CV), and 3) the commercial vaccine diluted 1:10 with phenolized saline (DCV). Volunteer groups of equivalent characteristics were established and bleedings were obtained prior to immunization and usually 1, 2, 3 and 6 weeks after each immunizing dose, and at 25 weeks immediately prior to re-immunization. All except one group were re-immunized at 26 weeks and appropriate blood samples were obtained through the remainder of the year. The tube-dilution vibriocidal assay using Ogawa and Inaba organisms was used to determine antibody titers.

The regime producing the best overall response on initial immunization was the use of CV in doses of 0.5 ml and 1.0 ml 1 week apart. Titers were significantly better than those produced by a single dose of the same vaccine. Two doses 4 weeks apart produced intermediate results. The second dose of this latter regime did not improve the group mean or produce a booster-type response, but did increase the titers of some individuals who responded poorly to the first dose. While the response to single doses of DCV was definitely inferior to the CV response, CRL which contained about twenty times more Inaba antigen and three times more Ogawa antigen than CV produced a response that was little better in respect to anti-Inaba titers and drastically inferior in respect to anti-Ogawa titers. Among the vaccines and dosage regimes tested no significant differences were found in the rate of decline of titers during the period of initial immunization.

Re-immunization at 26 weeks produced group mean peak titers that were no higher and usually lower than peak titers obtained on initial immunization, although antibody levels at the end of the year usually were somewhat higher than at the end of the initial immunization period. Persons showing good initial responses tended to respond more poorly to re-immunization. Small-dose initial immunization (DCV) followed by normal dose re-immunization (CV) had no advantages and double-dose re-immunization produced no improvement. On re-immunization with CRL, the anti-Inaba response was superior to that from CV but considering the large difference in antigen quantity the improvement was not notable.

Summary (continued)

The suppression of anti-Ogawa response with CRL seen at initial immunization was relieved upon re-immunization. The use of CV in two doses a week apart followed by re-immunization with the same vaccine produced vibriocidal titers that exceeded those produced by a single dose of CRL (as used in Pakistan) throughout the full year of the study. Persons receiving CRL as a single dose with no re-immunization maintained elevated and relatively constant titers between the 25th and 52nd week in the absence of any further identifiable antigenic stimulation.

Forty-seven percent of the 173 volunteers in this study had vibriocidal antibody prior to immunization, with at least 32% having no histories suggesting possible contact with cholera antigens. The occurrence of these natural antibodies was associated with improved responses to cholera vaccines and most sera tested were passively protective for mice.

To the extent that antibodies detectable in the vibriocidal test may be associated with immunity, the results of this study suggest that vaccines of normal and balanced potency may be useful in producing immunity of practical significance in adults who have resided in areas where cholera is not endemic.

FOREWORD

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In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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INTRODUCTION

Although field trials were critically necessary for demonstrating the basic effectiveness of cholera vaccine, they are not well suited for research on the optimization of conditions for employing an immunizing agent. With the low case rates that exist, the groups to be immunized must be so large as to be relatively impractical for the simultaneous evaluation of multiple variables. In addition, any studies involving natural infection must be performed in endemic areas where both natural contact with *Vibrio cholerae* and previous vaccination make assessment of effects difficult, particularly as the results may apply to populations in nonendemic areas. Populations in endemic areas are prone to reflect the history of cholera contact in their particular areas and several studies have demonstrated that adults in endemic areas have relatively high levels of naturally acquired cholera antibody. This situation may obscure vaccine effects which in other populations might be quite significant.

A serological study now has been completed using human volunteers in the United States to obtain comparative data concerning a variety of parameters of cholera immunization. These include the effects of antigen quantity, dosage interval, and pre-existing antibody upon responses to initial immunization; and in addition the effects of both initial immunization and re-immunization procedures upon responses to re-immunization.

The principal measure of antibody response that has been used in this study was the titration of vibriocidal antibody. Although it is difficult to conceive of the complement-dependent vibriocidal activity of serum as being the mechanism of vaccine-induced protection of humans against cholera, antibodies which are detectable by the vibriocidal test seem to be associated with immunity, at least to a sufficient degree to justify a working hypothesis that antigens and immunization procedures which improve the vibriocidal antibody response may have a favorable influence upon immunity to cholera.

MATERIALS AND METHODS

Volunteers. The human volunteers used in this study were inmates of the Ramsey Unit of the Texas Department of Corrections, and were males between the ages of 21 and 35 years. Pre-immunization bleedings were obtained from each individual and the various groups that were created in this study were balanced in terms of the heights and frequencies of pre-immunization vibriocidal antibody titers. The groups were similar in respect to race, height, weight, age, and the frequency of military service.

Vaccines. Three bivalent cholera vaccines were employed in this study. One was the vaccine that had produced significant protection in the Pakistan field trials of 1963 and 1964 (1). The vials of vaccine used in this study had been refrigerated continuously at the Division of Biologics Standards of the National Institutes of Health. This vaccine has been designated "CRL" in this report*. The second was a commercial vaccine meeting the present standards of the National Institutes of Health. This will be referred to as "CV"**. The third vaccine was prepared by making a 1:10 dilution of CV in phenolized saline. This diluted commerical vaccine has been designated "DCV". Table 1 shows the relative potencies of CRL, CV, and DCV, in comparison with the Ogawa and Inaba reference vaccines of the National Institutes of Health as determined by mouse-protective tests performed on CRL and CV, according to the method of Feeley and Pittman (2). The relative potencies of the three vaccines listed in Table 1 are based upon the use of equivalent volumes of each vaccine. However, CV and DCV were used in this study at 0.5 ml and 1 ml volumes, and CRL was used at 0.4 ml. Therefore Table 2 is provided to indicate the amount of either the Ogawa or Inaba reference vaccine that would contain the mouse-protective antigenicity that would be equivalent to the injected volumes of the various vaccines that were used in this study. The Inaba antigen potency varied over an approximate 200-fold range between 0.5 ml of DCV and 0.4 ml of CRL, whereas the Ogawa potency varied over a 24-fold range between these two vaccines.

Plan of Study. Table 3 is a flow chart demonstrating the subdivision of the initial pool of volunteers into the groups and ultimate subgroups of this study, and indicates the injections and bleedings that each received. The study was initially designed to be concluded at the end of the 32nd week (6 weeks after re-immunization). However, since 102 of the original 173 volunteers were available at the 52nd week an additional bleeding was taken to permit the analysis to cover two full immunization cycles.

For initial immunization, Groups 1, 4, and 6 received single doses of CV, DCV, and CRL respectively. Group 2 received two doses a week apart and Groups 3 and 5 were given two doses of CV and DCV respectively 4 weeks apart. After the bleeding of the 25th week, Groups 1, 3 and 6 were divided into subgroups A and B, the distribution being made to achieve approximate equivalence of vibriocidal titers between the subgroups in each respective group. Re-immunization was initiated at the 26th week for all groups and subgroups except 6B, to which no further injections were given. All groups except 6A and 6B received re-immunization with 0.5 ml of CV. Group 6A received 0.4 ml of CRL, and the uninjected Group 6B continued on to serve as a control group to represent the single-dose injection procedure that was used with this vaccine in the Pakistan field trials of 1963 and 1964. Groups 1B and 3B were re-injected 1 week after their first re-immunization dose, thereby becoming groups that could be compared to their subgroup A counterparts to determine the effects, if any, of a "double booster" re-immunization procedure.

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* Eli Lilly and Co. Lot #789864

** National Drug Co. Lot #6390

The design of this study permitted the pooling of group data in the early weeks of the experiment to bring more individuals to bear upon certain comparisons. As a minimum, bleedings were obtained before the initial immunizing dose and 1, 2, 3, 6 and 25 weeks thereafter. Following re-immunization, bleedings were obtained at 1, 2, 3, 6 and 26 weeks. For purposes of additional comparisons extra bleedings were obtained from certain groups as indicated, and a wide variety of internal comparisons were possible based upon the sampling plan that was employed. With minor exceptions, all injections were administered by means of an electrically operated jet-injector.

Losses of volunteers were unavoidable in a study of this type principally because of parole or transfer. The numbers of volunteers in each group or subgroup at certain key points in the study are given in Table 4. Our objective of having at least fifteen volunteers in each group through the originally-planned 32 weeks of the study was achieved.

Vibriocidal Antibody Titrations. Vibriocidal antibody titrations were performed by a tube dilution method similar to that described by McIntyre and Feeley (3). V. cholerae strains V-86 and 0-41 were the Inaba and Ogawa test strains used in these studies. Doubling dilutions of serum were employed in the test and the endpoint was considered to be the location in the test (either a tube or an interpolated value between tubes) where approximately 50% growth inhibition occurred. At the beginning of this study a standard series of serum dilutions was established, beginning at 1:2.5. It was therefore possible to express all results in terms of tube numbers, which then represented a progression based upon a dilution logarithm to the base of 2. Geometric means could then be calculated using endpoint tube numbers in direct arithmetic computations. In these studies the vibriocidal titer was the endpoint serum dilution calculated to include the addition of both cells and complement. Although 1:2.5 was the dilution of the initial tube on all pre-immunization bleedings, 1:20 (tube 4 of the standard series) represented the initial dilution of all post-immunization samples.

Since more than 4,000 vibriocidal assays were required, certain "mechanization techniques" were employed. With few exceptions, all serum dilution blanks and all reagent additions were dispensed with prechecked Brewer automatic pipetting machines, and serial serum dilutions were made with an Aminco automatic diluter capable of diluting eight sera simultaneously. Although each day's testing included a reference serum titration at the beginning and at the end, it was considered desirable to compensate further for possible day-to-day variations. Therefore serum samples were accumulated, and on any single day a series of at least three consecutive serum samples from each person were assayed together, and samples from an approximately proportionate number of individuals from each group were tested.

Statistical and Computational Procedures. Vibriocidal titers against both Ogawa and Inaba cells were recorded on punch cards, which served as the input for an IBM 1800 Computer. Since all of the vibriocidal data for one person could be accommodated on a single card, the development of comparisons and the calculation of various group statistics were greatly facilitated. All geometric mean vibriocidal titers calculated in this study were accompanied by the simultaneous computation of the Standard Deviation, and Standard Error for the group. Differences between means were tested for significance by the application of Student's t-test and were considered to be significant if values for P were equal to or less than 0.05. Appropriate statistical recognition was given to the fact that some group comparisons were made on the basis of paired data.

The volunteers in the various groups of this study were randomly located through thirteen wings of a large prison. Computer-generated address lists and procedure schedules contributed greatly to the orderly handling of injections, bleedings and payments to volunteers.

RESULTS

Because of the relatively large number of variables investigated in this study, it has been deemed advisable to consider separately the information dealing with initial immunization and that bearing upon re-immunization. These terms have no essential relationship to primary and secondary immunization as used in precise immunologic terminology.

Initial Immunization

The most controllable variation in initial immunization with cholera vaccine is in the number and spacing of the administered doses. In this study, the effects of these factors have been investigated through the use of a single commercial vaccine that was used undiluted, and diluted 10-fold (CV and DCV). Current practices have been followed in that the first or single dose of each vaccine was 0.5 ml and the second dose was 1.0 ml. Vibriocidal antibody responses were measured in groups of volunteers receiving CV as a single dose (Group 1), two doses a week apart (Group 2), and two doses 4 weeks apart (Group 3). DCV was given as a single dose (Group 4) or two doses 4 weeks apart (Group 5). The mean titer curves that were calculated from the responses of these five groups of volunteers are given in Fig. 1. The best response throughout the full period of initial immunization was obtained with two doses of CV given 1 week apart. This curve was significantly higher than that obtained from a single dose of CV at all points during the whole initial immunization period. The response to two doses of this vaccine given 4 weeks apart was consistently below the curve obtained with two doses a week apart, but these differences did not achieve statistical significance after the 4th week. In comparing the response to a single dose of DCV with that obtained from two doses given a month apart, a statistically significant difference was found only in the anti-Inaba response measured at 6 weeks. These curves show that whereas a second dose of vaccine administered 1 week after the first, when antibody titers are still rising, produced stimulation to a higher peak (Group 2), the same dose of antigen given 4 weeks after the initial injection, when the maximum response to the first dose had been passed, resulted in relatively minor stimulation (Group 3) even though the second dose was double the amount of the initial dose. Cnly in the case of the anti-Inaba response to DCV did the mean titer, following the second dose, equal or slightly exceed the initial peak (Group 5). In the adult volunteers that comprised these groups there is no evidence whatever of a secondary or booster response following the 4-week injection.

With each group of volunteers, serum samples were obtained 6 weeks after the antigen injections, as well as 25 weeks after the initial injection. The rate of decline of group mean titers may be judged from the slope of the lines from 6 weeks after the single doses or 6 weeks after the second dose, to the 25th week. No significant differences in slope were found among any of the ten curves in Fig. 1, indicating that within the limits of the conditions that were studied, neither the amount of antigen administered, the number of doses given, nor the differences in dosage interval had an effect upon the rate at which the group mean antibody titers decreased after initial immunization.

While the use of mean vibriocidal titer curves is a method of characterizing the dynamics of group changes, the individuals who respond poorly to immunization are objects of special concern, since in any group at risk they might be considered to be the persons most likely to become infected. Therefore these data were examined to determine whether the most effective of the multiple-dose regimes had particular effects upon the frequency of relatively low titers. The distribution of individual titers at 2 weeks in Group 2 (two doses of CV a week apart) was compared with the distribution in Group 1 (single dose). In both cases this was the time of maximum group response. Fig. 2 shows a cumulative percentage frequency plot for both the anti-Ogawa and anti-Inaba responses. Curves of this type indicate the percentages of each group (abscissa) having titers at or below the titer values designated on the ordinate. These curves demonstrate that the single dose method of immunization produced a relatively greater percentage of lower titers than did two doses given a week apart. This is particularly notable in respect to the anti-Inaba response. A similar effect was demonstrated in a different manner in Group 3 which received two doses of CV 4 weeks apart. This interval was sufficiently long that a separate rise in titer (if it occurred) could be detected after the second injection. Therefore the peak response following the first dose may be compared with that following the second dose. Among the sixteen persons representing the approximate lower half of the initial titer distribution, there were two who had improved (three tubes or more) anti-Ogawa titers and five who had improved anti-Inaba titers. Of the 22 persons in the upper half of the initial distribution, none showed improvement in anti-Ogawa titer, and only one had an increased anti-Inaba response. These results suggest that a certain few persons, mostly in the lower portion of the initial titer distribution, are stimulated to better responses by a second dose of antigen. This further stimulation of "poor responders", while in no sense meeting the definition of a booster response, may be the most significant effect of the second dose upon initial immunization of adults with cholera vaccine.

The effects of antigen quantity upon vibriocidal response can best be demonstrated with the results obtained with Groups 6, 1 and 4, whose members received single doses of CRL, CV. and DCV respectively. Based upon mouse-protection titrations, the Inaba antigen activity of CRL in the dose given was twenty times greater than that for CV, and the activity of DCV was ten times less than CV. Therefore these groups demonstrated the responses to an approximate 200-fold variation in Inaba antigen, with two groups (1 and 6) receiving amounts of antigen bracketing the range of many vaccines in current use. Fig. 3 demonstrates the highly significant superiority of the anti-Inaba response to CV over DCV. However, although the response to CRL was numerically superior to that from CV, this difference did not achieve statistical significance at any point within the whole initial immunization period. Within the first three weeks of these experiments, Groups 1 and 3, and Groups 4 and 5 are identical. A similar analysis covering this period, using the increased numbers of data obtained by the combination of these groups. together with the data from Group 6, demonstrated the same effects.

Fig. 4 shows the cumulative percentage curves of each of the groups (Groups 1 and 3, 4 and 5, and 6) having peak responses (regardless of week of occurrence) at or below the indicated titers. The titer distributions resulting from the administration of CV and CRL are practically the same except for the diminution of the frequency of lower titer responses when CRL was administered. The relatively small improvement in the serological response that was produced by the very large amount of Inaba antigen that was in CRL, appears to be a finding which may have much significance in the future development of cholera vaccines.

Fig. 5 gives the mean titer curves of anti-Ogawa response, following the administration of the same three vaccines (CV, DCV, and CRL) to Groups 1, 4, and 6. As with the anti-Inaba response in the same groups, the mean anti-Ogawa titers induced by DCV were lower than the CV responses at all points throughout the initial immunization period. These differences were statistically significant at all sampling times except 6 weeks. However the anti-Ogawa response induced by CRL was surprisingly poor and significantly inferior to the response produced by CV at all points. The cumulative percentage frequency data in Fig. 6 demonstrates that CRL induced a considerably greater number of lower titers than did either CV or DCV, even though the administered dose of CRL contained more than twice the anti-Ogawa mouse protective activity of the CV dosage and more than twenty times the potency of the DCV dosage. There appears to be a marked suppression of the anti-Ogawa response to CRL. If this is related to the large amount or unusual character of the Inaba antigen which CRL contains, these results suggest new factors that require further investigation in respect to the development of bivalent cholera antigens.

Since CRL is a vaccine of field-proven effectiveness against Inaba cholera, the anti-Inaba serological responses produced by this antigen have particular value for comparative purposes. CV administered in two doses 1 week apart (Group 2), has been found in this study to produce serological responses on initial immunization that were the best of any of the regimes that were investigated. Fig. 7 presents a direct comparison between the anti-Inaba mean titers produced by a single dose of CRL, and CV administered in two doses. The mean vibriocidal titers produced by these two vaccines did not differ significantly at any point, and CV was numerically superior at all points beyond the first week.

The distribution of individual titers not only at their peaks, but also at the end of the immunization period, is an important basis for comparison. Fig. 8 shows the cumulative percentage frequency distribution of peak vibriocidal titers regardless of week of occurrence and the distribution that obtained at the 25th week, 1 week prior to re-immunization. CV produced anti-Inaba titer distributions that were at least as favorable as CRL both at the beginning and the end of the initial immunization period.

Fig. 9 provides a direct comparison between the mean anti-Ogawa titers produced by CV and CRL. The highly significant difference in anti-Ogawa response resulting from the poor response to CRL is readily apparent. A consideration of the data summarized in Figs. 7, 8. and 9 suggest strongly that serological responses that are equal or superior to those obtained with CRL, can be induced with two doses of commercial vaccine of ordinary, balanced potency.

Re-Immunization

As indicated in Table 3, Groups 1, 3 and 6 were planned to be subdivided into A and B subgroups at 26 weeks when re-immunization would be performed. The 25-week bleedings of volunteers in these groups were titrated for anti-Ogawa and anti-Inaba vibriocidal activity immediately after collection, and individuals were assigned to the subgroups using the higher of the two vibriocidal titers as the basis for distribution. Therefore at the 26th week when re-immunization was initiated, all of the numbered groups and the lettered subgroups contained approximately the same numbers of volunteers (Table 4). All groups except 6B received some form of re-immunization (Table 3). In order to permit "paired data" comparisons to be made, the values provided by volunteers who were lost from the study prior to the time of re-immunization were excluded, and the results of initial immunization were recalculated using only those volunteers who were still available at the time of re-immunization. Table 5 characterizes the immunization history of the various groups and gives the maximum mean vibriocidal titer obtained in each group following both initial immunization and re-immunization. These are expressed as tube numbers in the standard series of dilutions which have been used in this study, and were calculated using the maximum titer reached by each individual after injection, regardless of the week of occurrence. The only instance in which there was a significant improvement in the maximum mean titer was in the anti-Inaba response of Group 4 where initial immunization consisted of a single dose of DCV, and re-immunization was given with CV. In all other groups where CV was used for re-immunization, the maximum mean titers were either about the same as or significantly lower than those which had been achieved on initial immunization.

Re-immunization with two doses given a week apart neither improved the relationship between initial immunization titer and re-immunization titer, nor did it produce re-immunization responses that were notably better than those obtained by a single dose re-immunization (1A vs. 1B, and 3A vs. 3B). This "double booster" technique apparently had no additional antigenic action.

Among the 86 volunteers who received initial immunization and re-immunization with CV, *increases* of three tubes or more over their initial immunization peak were shown in the anti-Ogawa titers twice, and in the anti-Inaba six times. In each instance, the initial immunization titer was found to have been in the lower third of the group. Among these same volunteers, *decreases* of three tubes or more from their initial immunization peak were observed in the anti-Ogawa titers eight times, and the anti-Inaba titers sixteen times. In all but two instances the initial titers were in the upper third of the group. This diminished response to re-immunization among individuals who had previously shown the better responses to initial immunization was not concentrated in any group having a particular immunization history, and may be an important point to consider in the development of recommendations concerning multiple re-immunization.

The re-immunization response in volunteers receiving CRL (Group 6A) may have special significance. The marked suppression of the anti-Ogawa response seen following initial immunization was considerably relieved after re-immunization, and the peak mean titers were equivalent to the best of the other groups. While the anti-Inaba titer improvement shown by this group following re-immunization did not achieve statistical significance, it was significantly better than all of the re-immunization responses produced by CV. Therefore, in respect to maximum mean titers produced on re-immunization, CRL was about equal to CV in anti-Ogawa response, and superior to it in anti-Inaba response.

These data do not provide support for a procedure of employing a small initial immunization dose followed by a larger re-immunization dose. Although this regime in Group 4 produced a significant improvement in the anti-Inaba maximum mean vibriocidal titer, comparing re-immunization with initial immunization, the re-immunization responses of Groups 4 and 5 that received low-level initial immunization were equal to or below all groups that received stronger initial immunization.

Groups 1A, 3A and 6A afford the best basis for comparing the relative rapidity with which maximum titers were achieved following initial immunization and re-immunization. Table 6 shows the average time required for individuals in these groups to achieve their maximum titers after injection. Except for the Ogawa response in Group 1A, all showed a significantly more rapid response following re-immunization. Differences were not great, however, and the relative rapidity with which maximum responses are reached, even on initial immunization with single doses, appears to be a notable property of cholera vaccine administered to adults.

Although the height and time of peak response are two characteristics which may be of assistance in describing responses to immunization, the value to which the titer falls within the period between antigenic stimulations may be of even greater importance. Table 7 presents the minimum mean vibriocidal titers that were obtained at the 25th week and 52nd week of this study using only data from volunteers who were available for the whole year. Although the maximum mean vibriocidal titers of these groups were, in general, equal or significantly lower following re-immunization, the minimum mean titers after re-immunization were equivalent or significantly higher than the minimum values after initial immunization. This suggests that the persistence of serum vibriocidal activity might be somewhat better following re-immunization than it was following initial immunization. The mean titers for Group 6B which received a single dose of CRL and no re-immunization are included at the bottom of this table for comparative purposes. It may be of some importance that all groups that received two initial doses of CV and re-immunization at 6 months (Groups 2, 3A and 3B) had minimum anti-Inaba titers that equaled or exceeded those of Group 6B, while also displaying anti-Ogawa titers that were superior to those obtained in Group 6B.

Group 2 which received two doses of CV a week apart followed by single dose re-immunization, has demonstrated both anti-Ogawa and anti-Inaba titers that have been consistently equal or superior to those produced in the other groups receiving CV or DCV. Fig. 10 compares the mean vibriocidal titer curves of this group and Groups 6A and 6B across the full year of the experiment. The values for these curves were derived only from persons available through the full year. In this figure, it is demonstrated that following initial immunization, the anti-Inaba response of Group 2 (CV) is equivalent to those of 6A and 6B (CRL), and the anti-Ogawa response is considerably superior. On re-immunization, the anti-Inaba response of Group 2 is somewhat less than that of 6A, but the anti-Ogawa responses of these two groups are essentially similar.

"Natural" Antibody. Many persons in cholera-endemic areas have vibriocidal antibody presumably from prior natural contact with cholera antigens. However the frequency, source and effects of similarly-reacting antibody in persons in non-endemic areas have not yet been clarified. Of the 173 volunteers in this study, 82 (47%) had detectable pre-immunization antibody. Of these, 41 and 12 had antibodies only for Ogawa or Inaba respectively, while 29 had antibodies reactive with both serotypes. Of this group, 56 had no history remotely suggesting contact with cholera antigens, and of the remaining 26 persons, where service in the Armed Forces or travel overseas made it impossible to exclude such contact, collateral information indicated that it was quite unlikely in many instances. Therefore it is considered that at least 32% of the persons in this study had detectable anti-cholera antibody of probable non-cholera origin. The mean titers for all positive pre-immunization sera were 1:53 and 1:87 for Ogawa and Inaba respectively.

Peak mean titers after initial immunization and re-immunization were calculated for persons with and without pre-immunization antibody in Groups 1 and 3 combined. While these groups were not completely identical, they had both received immunization with CV, and their overall group responses had been very similar. In this analysis, individuals having pre-immunization titers responded significantly better to initial immunization than those who had no detectable antibody. However after re-immunization at 26 weeks, when practically all persons had detectable antibody against both serotypes, the difference in response practically disappeared. Similar results were found in analyses of other groups receiving either DCV or CRL.

The pre-immunization sera of fourteen persons having vibriocidal antibodies but no histories suggestive of prior contact with cholera antigens, were tested in passive protection tests in mice. All of ten sera having anti-Ogawa vibriocidal antibody were protective against

Ogawa challenge. However, of the seven sera having anti-Inaba vibriocidal activity, three showed anti-Inaba protection, while the others did not at the concentrations that were employed. Serum samples from other volunteers, both prison inmates and medical students, are becoming available from our other cholera antigen studies, and they are showing a frequency of natural antibody similar to that described above.

DISCUSSION

Cholera organisms vary in the number, kinds and amounts of the antigens they contain. The protective antigen obtained from Ogawa cells is demonstrably different from the equivalent Inaba antigen in chemical and physical properties (4) even though sharing certain immunologically related specificities (5). Even a single antigen may induce the formation of a dispersion of immunoglobulins having different specificities, avidities and tissue permeabilities. Although the participation of some of these globulins in protection against cholera is not seriously questioned, there is no clear indication concerning which ones are involved or what the mechanism may be which protects against intralumenal infection. The prior immunologic experience of the host in respect to the same or related antigens influences the extent and specificity of his response to injected antigen; and relative immunologic maturity also affects responsiveness. Studies in human volunteers, particularly those under institutional control, seem to be quite suitable for the investigation of many of these variables, provided the number of volunteers is sufficiently large to minimize the influence of individual variations.

This study was designed to have its principal application to populations of male adults of "military age" residing in areas where cholera infection does not occur. Therefore the volunteer group was restricted to males between the ages of 21 and 35. While this has probably resulted in an improvement in homogeneity, it places limitations upon the general inferences which may be drawn from the results of this study. Consequently any extension of the findings in this study to endemic area populations or to children must be made with appropriate caution.

In respect to individuals who reside in areas where cholera is not endemic and who then become "at risk" either through the invasion of the disease or their own travels to an endemic area, an optimal procedure for initial immunization is of major importance. These studies have shown that a single dose of any of the vaccines employed produced a considerable rise in vibriocidal titer in most individuals. However when a second dose was given 4 weeks later most persons were not stimulated to higher antibody levels than they achieved following their first dose. Both the anti-Ogawa and anti-Inaba responses were similar in this respect and this behavior was seen with both CV and DCV, differing 10-fold in concentration. It is therefore clear that no booster effect has been observed following the 4-week injection and it would appear that the chief value of a second injection at 4 weeks may be to prolong the antibody response slightly and to produce additional stimulation of a relatively few individuals who had responded somewhat poorly to the first dose. The further stimulation of "poor responders" may be of considerable epidemiological significance if such persons are assumed to be the more susceptible members of the population. Mosley (6) in studies of admission titers of hospitalized cases, has presented evidence supporting this assumption.

The most effective procedure for initial immunization was the use of commercial vaccine in two doses, 1 week apart. In this regime where the second dose was administered while serum antibody titers were still rising, there was a marked improvement over single dose immunization both in group mean titers and the frequency of relatively low titers. This method for initial immunization seems to be practical under most conditions where cholera immunization with two doses could be employed. It is noteworthy that none of the procedures that were used resulted in any significant differences in the rate with which group mean titers declined after initial immunization, and the titers at the end of the initial period were, in general, proportional to the initial peak titers that were produced. While this situation may be true only for plain whole cell vaccines, it suggests the possibility that at least these immunizing agents may be tested in human volunteer experiments of relatively short duration with reasonable assurances that the peak responses may be indicative of their relative antibody-producing potential.

The effect of antigen quantity upon serological response is a factor of major importance, both immunologically and economically. While it is frequently assumed that the effectiveness of a vaccine may be improved by increasing the amount of antigen which it contains, it is also known that there is an upper limit to this response. These studies employed vaccine dosages which varied 200-fold in their Inaba mouse-protective antigenicity; and two of the vaccines (CV and CRL) bracketed the range of antigenic potencies of vaccines currently produced in this country. While the group mean vibriocidal responses to CV were considerably improved over the responses to DCV, a further 20-fold increase in potency resulted in remarkably little further improvement. In addition, CRL produced anti-Ogawa group mean titers that were far below what would have been expected on the basis of its anti-Ogawa mouse protective activity. These data suggest that at the concentrations of Inaba antigen that are included in vaccines of usual potency, the responses of individuals may be approaching their maxima and that further increases in titer can be accomplished with plain whole cell vaccines only through the use of considerably larger amounts of antigen. Insofar as the production of vibriocidal antibody may be related to practical immunity, these data suggest that the use of plain vaccines of increased potency, within the range of practical achievement, may not have much favorable effect upon either the height of the initial response or the subsequent rate of decline of antibody titers. Conversely, however, it is clear that the use of vaccines of lower potency than CV carries the risk of a decreased antigenic response, and the increased frequency of relatively poor titers.

While these studies provide no clear contraindications to increasing the antigen concentrations in cholera vaccine, the unexpectedly poor anti-Ogawa response to CRL at least suggests that some form of balance between Ogawa and Inaba content may be desirable. Hornick et al. (7) have obtained somewhat similar results with the same vaccine and at present it is not known whether this is a special property of the vaccine or is a general manifestation of antigen imbalance. Since Watanabe et al. (5) have demonstrated a shared as well as a specific reactivity to purified Ogawa and Inaba protective antigens, competition for the same immunocompetent cells must be considered. This problem requires further investigation.

Although evidence has been presented that initial immunization of adults with two doses of CV a week apart is superior to single-dose immunization, even single doses of 0.5 ml produced a relatively prompt response of considerable magnitude in almost all persons. These studies offer serological justification for the use of a single-dose immunization procedure for adults in preference to two smaller doses a month apart under emergency conditions or when supplies of vaccine are limited. However, data of Mosley et al. suggest that a second dose has considerably more significance in the initial immunization of children (6).

There are many practical implications to the observation of a good response following two doses of vaccine a week apart. These studies do not indicate whether a shorter interval or a smaller second dose can produce comparable responses. It is believed that such variables could be tested with relatively short-term volunteer studies, employing longer studies to investigate only the most promising regimes.

In these studies 47% of all volunteers were found to have detectable antibodies against one or both cholera serotypes, with anti-Ogawa antibody being more frequent. Most of these persons had no history of exposure to cholera antigens and it may be presumed that the stimulus for this antibody production has come from other environmental organisms having related antigens. Regardless of the source, however, individuals having this prior experience tended to respond better to initial immunization than did those without detectable antibody. Since the groups in this study were balanced in terms of pre-immunization vibriocidal antibody, this factor probably has not influenced the intergroup comparisons. Furthermore, it is believed that the existence of natural cholera-reactive antibody is a general property of populations in non-endemic areas, since it has been noted by others both in the United States and in Czechoslovakia and has been found in our own studies with about equal frequency among medical students as well as prison inmates. The considerably lower frequency of detectable titers found by Mosley et al. (8) in Czechoslovak serum samples was probably the result of a somewhat less sensitive testing technique. Since persons having preexisting cholera-reactive antibody represent a normal and significant portion of the adult population in non-endemic areas, it is believed that they should not be excluded from any studies purporting to define the immunological respone of a non-endemic population. However, such persons should be detected and their influence distributed equally in any groupings that may be developed.

These studies have been more successful in defining the limitations of re-immunization than in revealing any superior procedures. Re-immunization peak titers, with one exception, were not improved over initial peak titers, and in most instances they were significantly lower. Neither small-dose initial immunization nor two-dose re-immunization was of any advantage. Although after re-immunization antibody titers rose more quickly and tended to remain at higher levels at the end of the period, these differences were not striking and did not suggest the occurrence of a booster-type response.

Individuals who demonstrated relatively high titers following initial immunization frequently had poorer responses to re-immunization, while those whose initial response was not so marked tended to approach their initial levels or to improve somewhat following re-immunization. The association of good initial responses with lessened response to re-immunization may have some importance in considering problems of multiple re-immunization. These studies extended only through a single re-immunization cycle. Other studies now in progress under Contract DADA 17-68-C-8152 will follow volunteers through several cycles and will investigate the effects of longer intervals between immunizations. The fact that the one group which received no re-immunization (6B) maintained an essentially unchanged mean titer from the 25th week to the 52nd week suggests that antibody production may tend to stabilize with time, and if the level can be made sufficiently high for most persons, fewer re-immunizations at longer intervals might be considered. If immunization is impairing the responsiveness of individuals to subsequent dose of cholera vaccine, intervals between injections that are longer than 6 months may be beneficial.

It is of special significance that the persistence of relatively high vibriocidal antibody levels that was seen in Group 6B occurred in the United States in an essentially closed population. Persistence of vibriocidal antibody titers at relatively high and constant levels has been noted in populations in endemic areas, and the role of subclinical contact with cholera organisms has been a subject of speculation. Group 6B was clearly not exposed to cholera organisms, and there is evidence that there was no unusual exposure to whatever antigens produced natural vibriocidal antibody in the United States. In the period near the end of this study, pre-immunization bleedings were obtained from 90 additional volunteers who lived in the same prison wings and ate in the same dining hall with the members of Group 6B. These new volunteers showed a frequency and range of levels of natural antibody that were similar to those observed approximately a year previously when this study was begun. Therefore it is suggested that the persistence of antibody production at elevated and relatively constant levels between the 25th and the 52nd week that was seen in Group 6B is not dependent upon continuing contact with cholera antigens, as might be postulated to occur in endemic areas. It is not known at present how long this antibody production will persist or whether vaccines other than CRL will produce this effect.

In this study differences in responses to various procedures have been considered to be "significant" if they cannot be reasonably ascribed to chance. Statistical significance does not necessarily imply epidemiological significance, and it is quite possible that differences in immunization procedures that resulted in significant serological differences may not produce significant differences in attack rates under the conditions of the usual field trial in an endemic area. In such areas the adult population has already had sufficient prior contact with cholera antigens that it is already significantly immune, and therefore does not represent a susceptible control baseline against which vaccine effects can be measured. This may lead to difficulties in assessing the degree and duration of immunity as they might apply to more susceptible populations drawn from non-endemic areas.

The possible significance of the findings in this report as they may apply to populations in non-endemic areas may be suggested by certain comparisons with serological data that have been obtained in East Pakistan by Mosley et al. These workers have shown that increasing group mean vibriocidal titers were associated with decreasing Inaba cholera case rates, whether the vibriocidal titers were measured following cholera immunization or were entirely the result of prior antigen contact in the control population. In addition, studies of familial contacts of index cases showed similar effects and demonstrated that a vibriocidal titer of 1:20 in the microtiter test was associated with a detectable decrease in the incidence of cholera (6). In both the vaccine-immunized and control populations, a doubling of the vibriocidal titer was associated with an approximate 50% decrease in case rate.

Although it is realized that in regard to the present study any interpretation of serological responses in terms of immunity must be entirely speculative, it was believed some effort in this direction might at least be of interest and possibly of assistance in developing working hypotheses for future studies. To make comparisons of serological responses possible between our studies and those in Pakistan, it was necessary to correlate vibriocidal titers as measured by the two laboratories. Workers from our laboratories with their own cultures and supplies carried on tube dilution vibriocidal tests at the Pakistan-SEATO Cholera Research Laboratory, while the personnel of that laboratory were performing the microtiter test on the same serum samples. Forty sera from this study and twenty from cholera cases in Pakistan were tested simultaneously and repeatedly by the two methods. Correlations between the two tests were found to be linear for both anti-Ogawa and anti-Inaba titerations. Taking into consideration the differences in conventions in reporting titers in the two laboratories, and the greater sensitivity of the tube dilution test, there was approximately a three-dilution difference, with a 1:20 titer in the microtiter test being equivalent to a 1:160 titer in the tube dilution test. Since the slopes of the correlation curves of the Ogawa and Inaba test were both approximately 0.8, the differences between the two titration methods expanded to about five dilutions at higher titers.

Because of the relatively few cases of Ogawa serotype cholera that have occurred in the Pakistan field trial areas in recent years, correlations between serology and immunity suitable for extension into the consideration of anti-Ogawa serology in non-endemic areas are largely lacking. Therefore consideration will be given only to data involving anti-Inaba responses.

The anti-Inaba mean vibriocidal titer of the total group of volunteers in our study prior to immunization was approximately 1:10 in the tube dilution test. This would be less than 1:2 in the microtiter test and is far below the mean titer of the most susceptible group (age 0-4, unvaccinated) which Mosley et al. (8) have reported. This group had a case rate of 170.4 per 10,000. Although in the Pakistan study the highest susceptibility was found in the youngest age group, there is ample historical evidence that susceptibility to cholera is not a special attribute of children and that in populations from non-endemic areas adults can be very susceptible. It appears to be a reasonable assumption that the volunteers in the present study were highly susceptible prior to immunization. Using the responses of Group 2 for purposes of illustration, after initial immunization the group mean titer rose from 1:10 to approximately 1:5120, and never went below 1:450 at any time during the full year of the study. If one applies to these data the observations of Mosley et al. that doubling of the vibriocidal titer was associated with a halving of the case rate, it would appear that the volunteers in this study may have had a very small fraction of their original susceptibility at the peak of their response and maintained a susceptibility that was considerably less than original throughout the year. To the extent that vibriocidal titers may be associated with immunity, these data also suggest that the level of anti-Inaba immunity produced in Group 2 would be expected to be observable under the conditions of exposure that existed in the familial contact studies reported by Mosley et al. The mean vibriocidal titers of this group remained continuously within the range of titers which were associated with decreased infection rates in the family contact studies.

The mean titers of the group showing the best overall response on initial immunization with CV were not significantly different from those produced by CRL, a vaccine of proven effectiveness even in susceptible children. Other groups immunized with DCV had mean titers that were as much as 4-fold lower. If a doubling of vibriocidal titer is associated with a 50% decrease in case rate, it is apparent that the magnitude of some of the differences in serological response produced by the various immunization procedures of this study might be expected to have practical as well as statistical significance.

Speculations concerning the relationship between anti-Ogawa serological responses and immunity to Ogawa cholera cannot be made at this time, since suitable serological studies have not yet been performed in endemic areas. It is to be hoped that all future vaccine field trials or epidemiological studies of cholera will be accompanied by thorough and simultaneous serological studies.

INVESTIGATIONS IN PROGRESS

This report has discussed antibody production primarily in terms of whole serum vibriocidal antibody titers. Further definition of these responses in terms of the proportion of activity that is attributable to the γM and γG immunoglobulins, and the pattern of the γA response are questions of major importance. A limited number of samples drawn from this study have been fractionated by column chromatography and are being studied by radial immune diffusion and antibody titration. In addition serum samples have been treated with anti-γM and anti-γG rabbit serum for the immunologic precipitation of the globulins, and subsequently tested for residual vibriocidal activity. The results of this work are not yet sufficiently satisfactory and extensive to be reported.

Live-cell agglutination tests are currently in progress on all sera of Groups 1, 2, and 6A for the purpose of studying the relationship between vibriocidal activity and agglutinating activity.

Several other analyses of vibriocidal antibody data remain to be completed. One of particular importance involves the question of the relationship between the circulating antibody titers immediately before re-immunization and the re-immunization response. Since the work planned under Contract DADA 17-68-C-8152 is a direct extension of the present work, the two studies will be cross-contributory. In addition, similar studies are currently in progress using single doses of an Inaba purified antigen and the experimental NIH Inaba monovalent vaccine, both of which are being used in field trials in East Pakistan at present. Since all of this work is being done in a closed population of the same specification the total results will broaden the findings of this study very considerably.

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	Anti-Ogawa	Anti-Inaba
NIH Ref. (Ogawa)	1.0*	--
NIH Ref. (Inaba)	--	1.0*
CRL	3.6	25.0
CV	1.17	1.01
DCV	0.12	0.10

* Set at unity

TABLE 1. Relative potencies of CV, DCV and CRL compared to NIH reference vaccines on an equal volume basis

---

		Reference Vaccine Volume	
Vaccine	Injected Volume	Ogawa	Inaba
CRL	0.4 ml	1.44	10.0
CV	1.0	1.17	1.01
CV	0.5	0.59	0.51
DCV	1.0	0.12	0.10
DCV	0.5	0.06	0.05

TABLE 2. Volume of NIH reference vaccine equivalent in mouse protective potency to the injected amounts of CRL, CV and DCV

	GROUPS								
Designation	1		2	3		4	5	6	
Vaccine	CV		CV	CV		DCV	DCV	CRL	
Initial N	39		20	38		20	17	39	
Week	PROCEDURES								
-2	B[+]		B	B		B	B	B	
0	0.5 ml[++]		0.5 ml	0.5 ml		0.5 ml	0.5 ml	0.4 ml	
1	B		B	B		B	B	B	
			1.0 ml						
2	B		B	B		B	B	B	
3	B		B	B		B	B	B	
4			B	B			B		
				1.0 ml			1.0 ml		
5				B			B		
6	B			B		B	B	B	
7			B	B			B		
10				B			B		
25	B		B	B		B	B	B	
	A	B		A	B			A	B
26-0*	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml**	0.5 ml**	0.4 ml	
27-1	B	B	B	B	B	B	B	B	
-		0.5 ml			0.5 ml				
28-2	B	B	B	B	B	B	B	B	
29-3	B	B	B	B	B	B	B	B	
30-4		B			B				
32-6	B	B	B	B	B	B	B	B	B
52-26	B	B	B	B	B	B	B	B	B

+ = Bleeding

++ = Injection of the indicated volume

* Weeks after re-immunization

** Vaccine CV injected for re-immunization

TABLE 3. Flow chart of the distribution of volunteers into groups and the injection and bleeding schedule for the groups

Week of Study	Group								
	1		2	3		4	5	6	
0	39		20	38		20	17	39	
25	34		18	35		17	15	34	
	A	B		A	B			A	B
26	17	16	18	18	17	17	15	17	17
32	17	15	18	18	16	17	15	17	15
52	14	10	14	11	10	9	11	12	12

TABLE 4. The number of volunteers in the groups at various times throughout the study

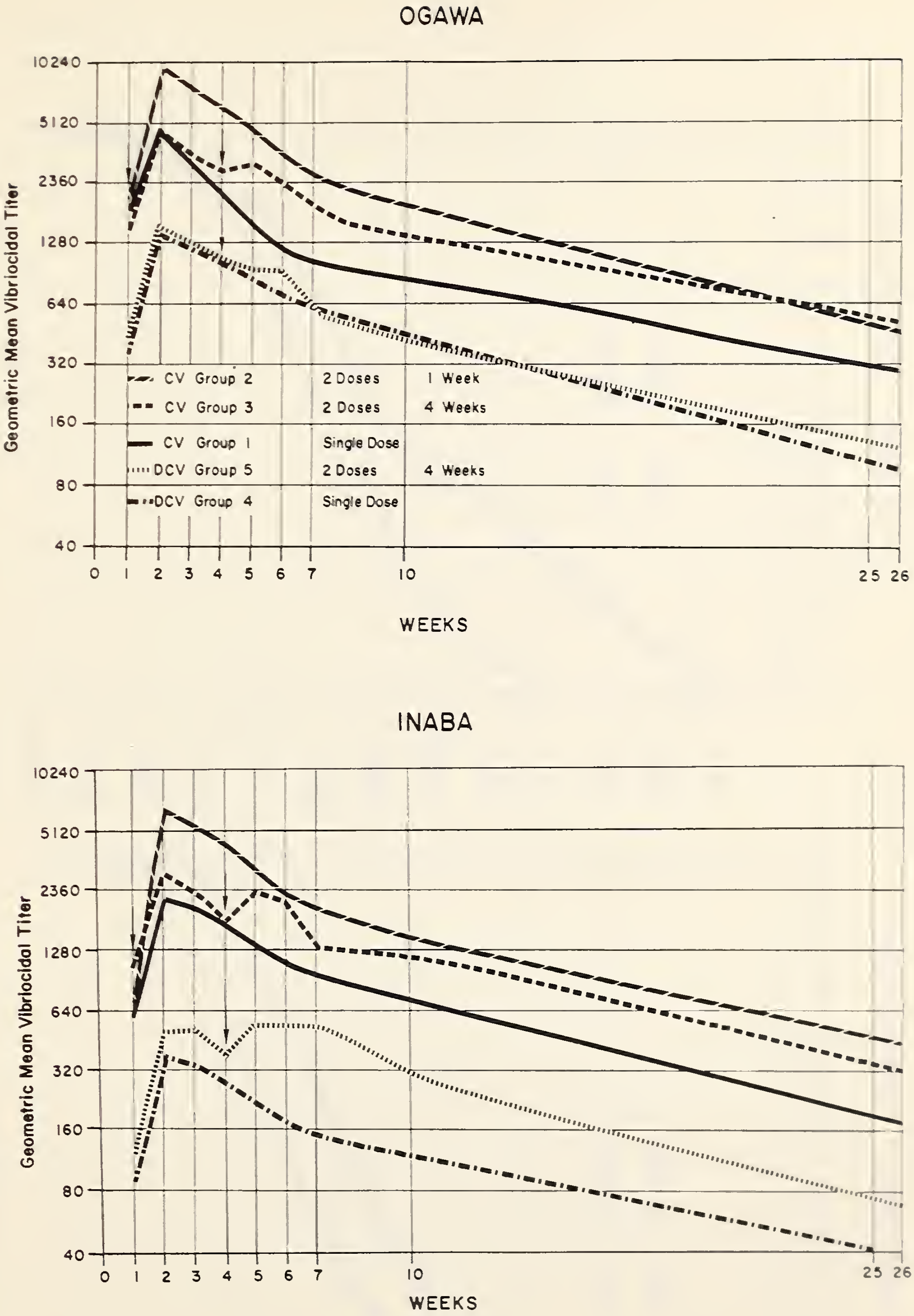

FIG. 1. THE VIBRIOCIDAL RESPONSES OF VOLUNTEERS TO ONE OR TWO INJECTIONS OF CV OR DCV

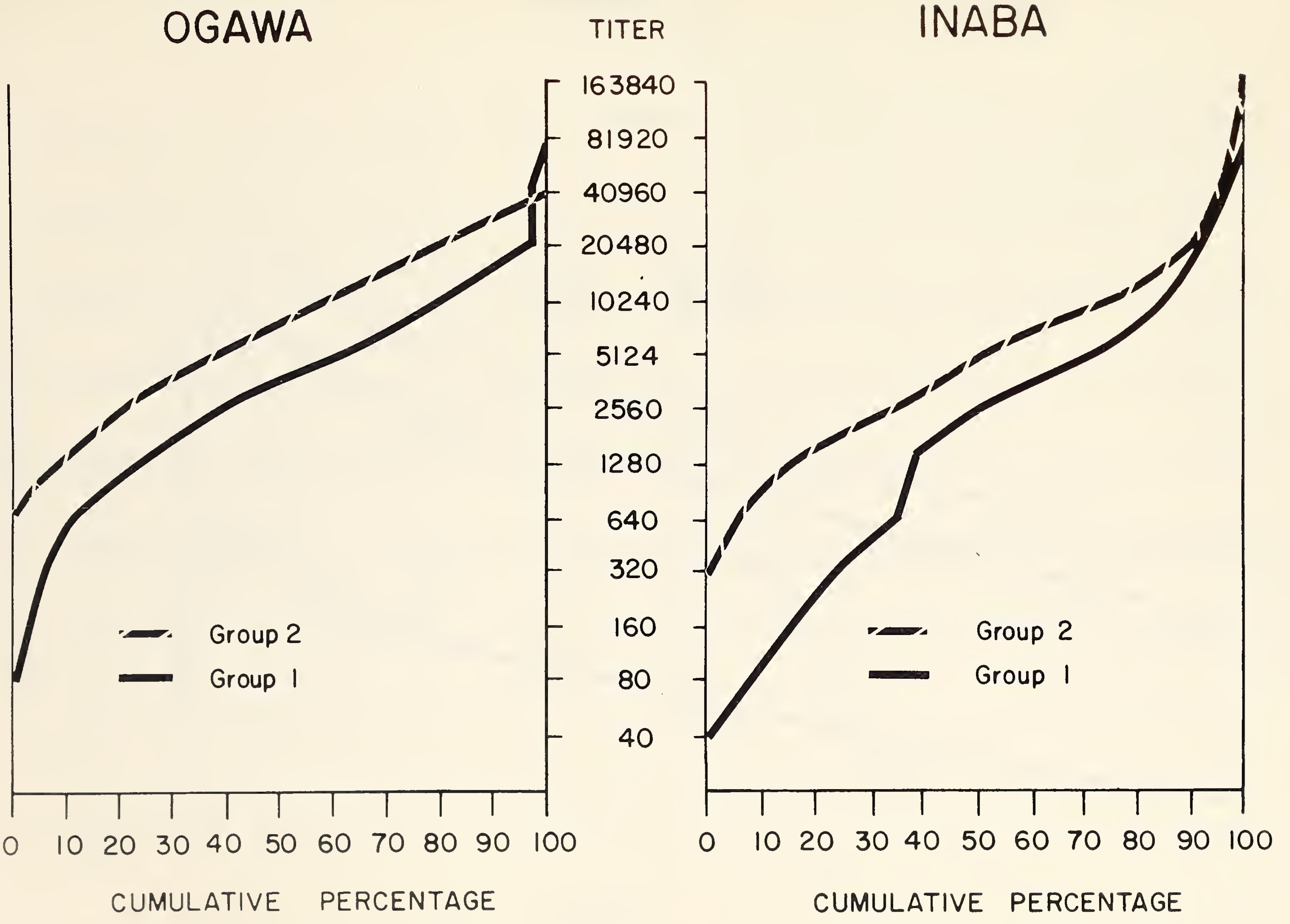

FIG. 2. CUMULATIVE PERCENTAGE DISTRIBUTION OF TITERS IN GROUP 1 (SINGLE DOSE) AND GROUP 2 (2 DOSES ONE WEEK APART) TWO WEEKS AFTER INITIAL IMMUNIZATION WITH CV

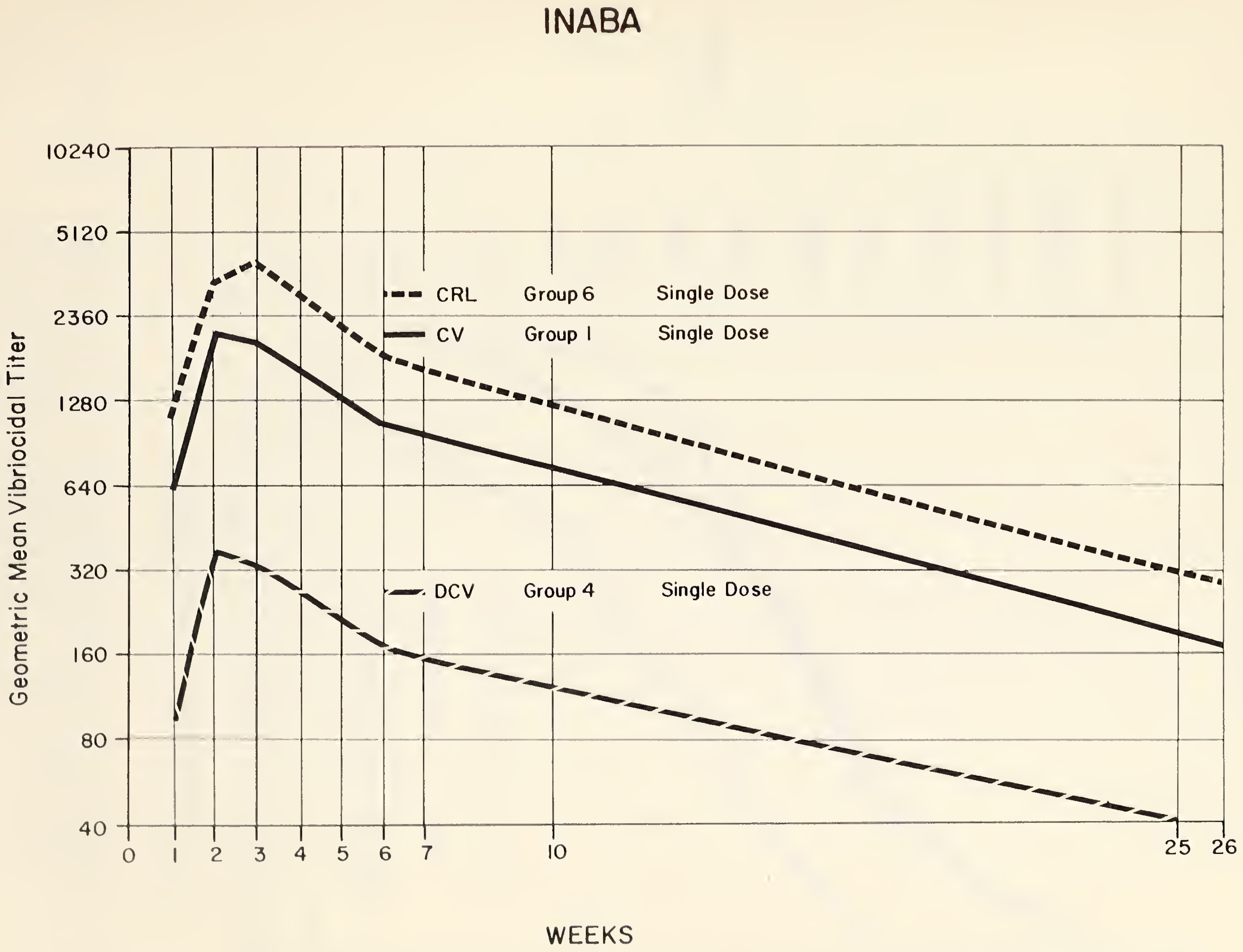

FIG. 3. THE ANTI-INABA VIBRIOCIDAL RESPONSES OF VOLUNTEERS TO SINGLE DOSES OF VACCINES DIFFERING IN MOUSE PROTECTIVE POTENCY

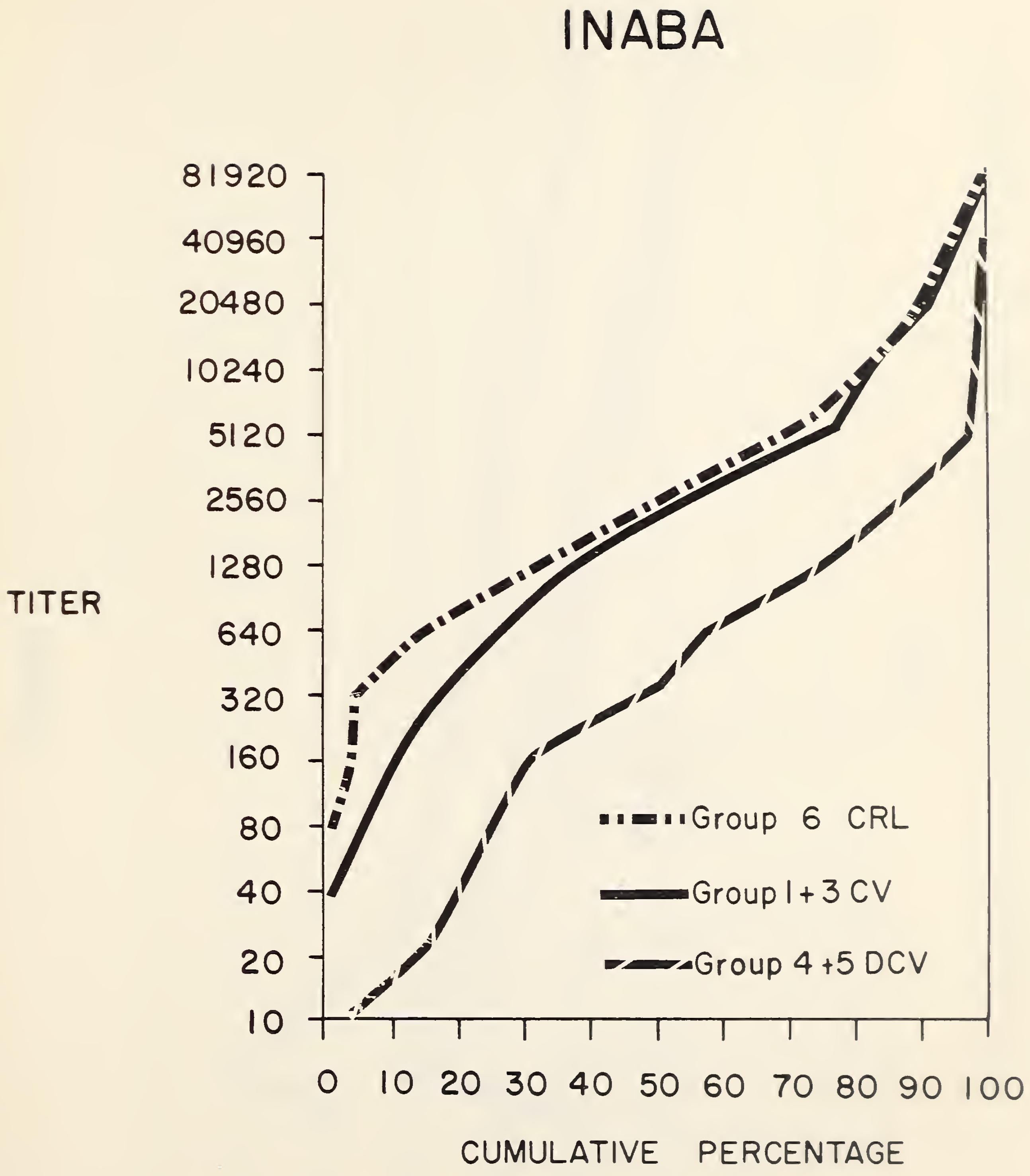

FIG. 4. CUMULATIVE PERCENTAGE DISTRIBUTION OF PEAK TITERS AFTER INITIAL IMMUNIZATION WITH SINGLE DOSES OF VACCINES OF VARYING ANTIGENIC POTENCY

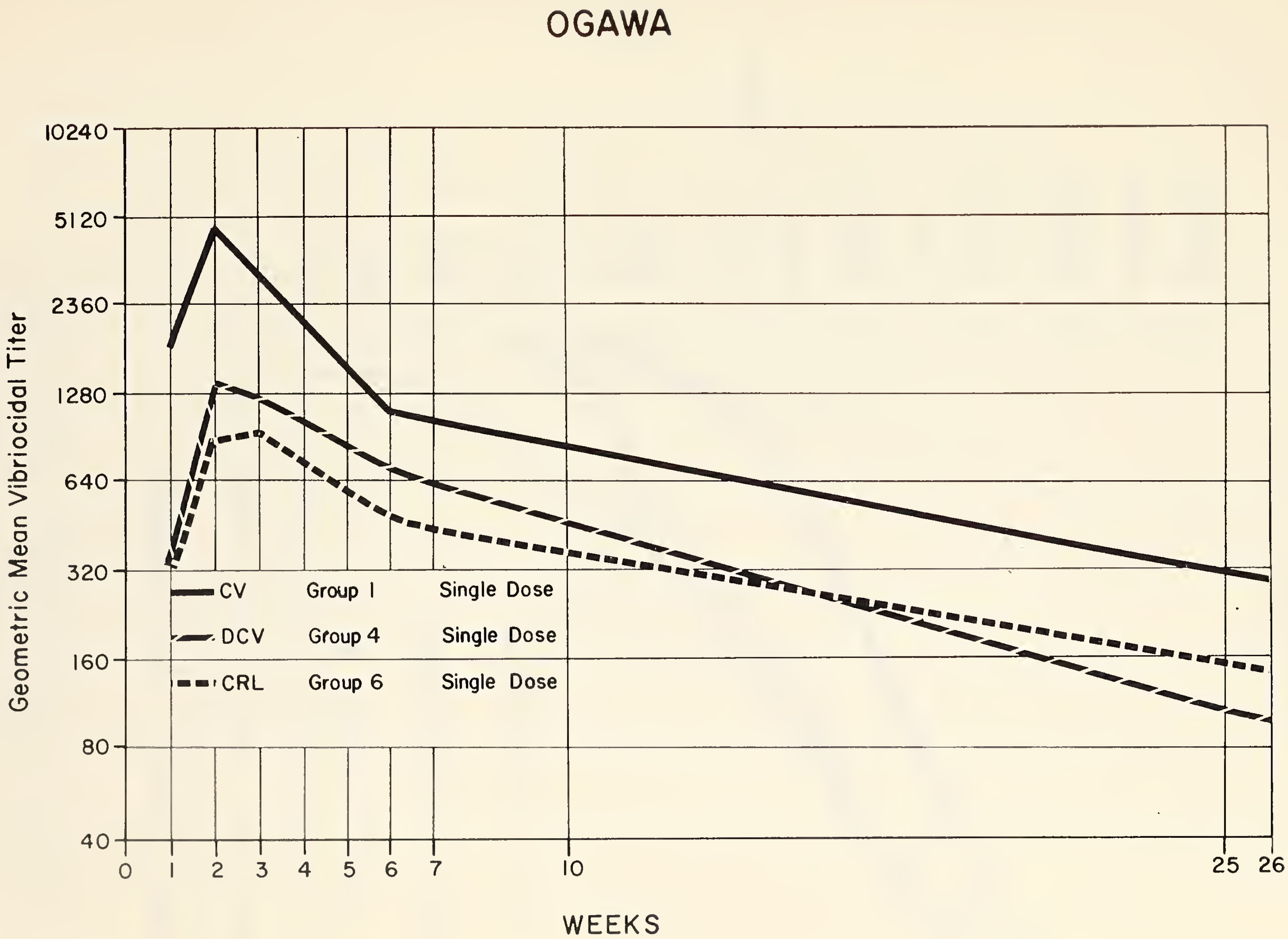

FIG. 5. THE ANTI-OGAWA VIBRIOCIDAL RESPONSES OF VOLUNTEERS TO SINGLE DOSES OF VACCINES DIFFERING IN MOUSE PROTECTIVE POTENCY

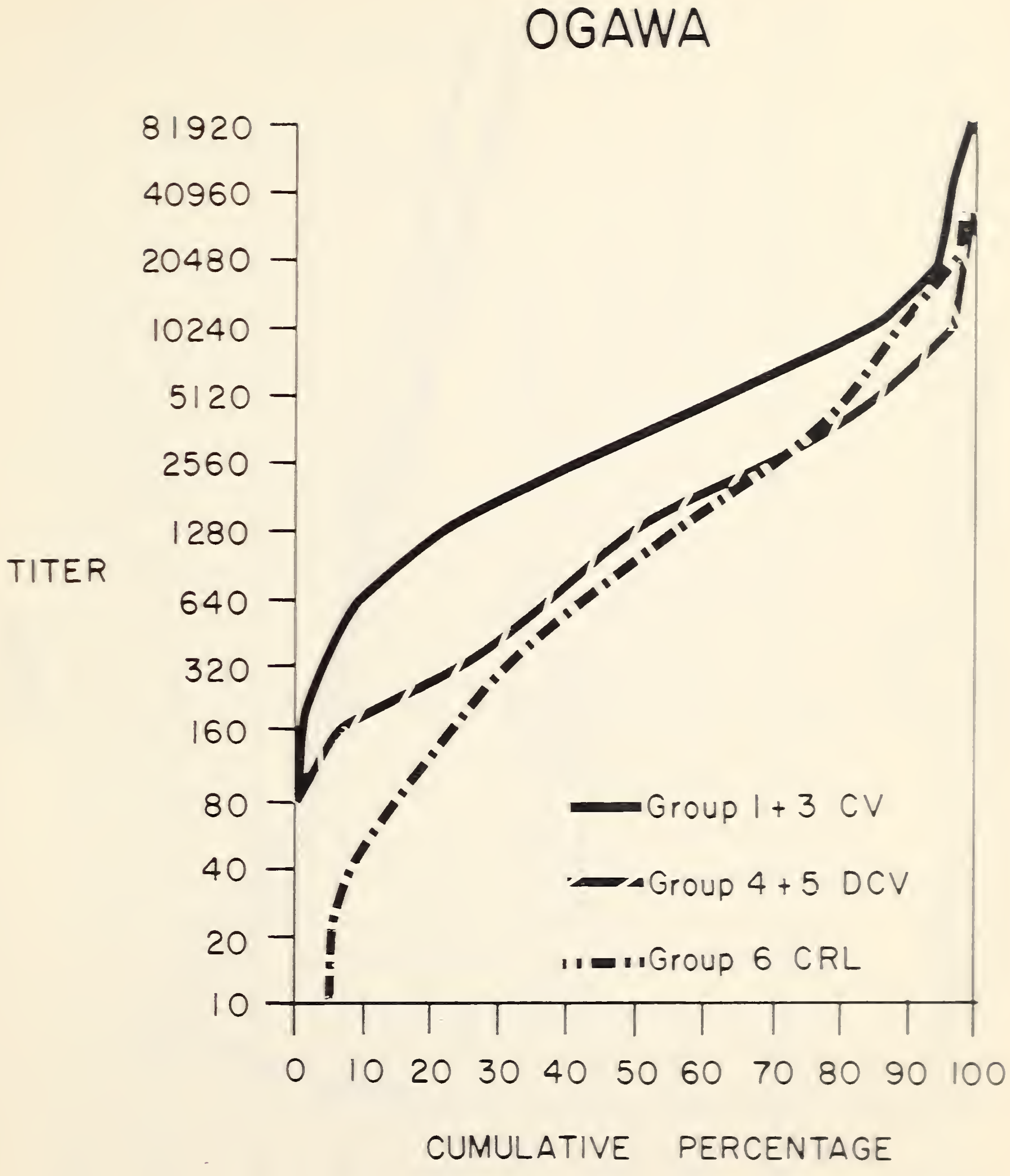

FIG. 6. CUMULATIVE PERCENTAGE DISTRIBUTION OF PEAK TITERS AFTER INITIAL IMMUNIZATION WITH SINGLE DOSES OF VACCINES OF VARYING ANTIGENIC POTENCY

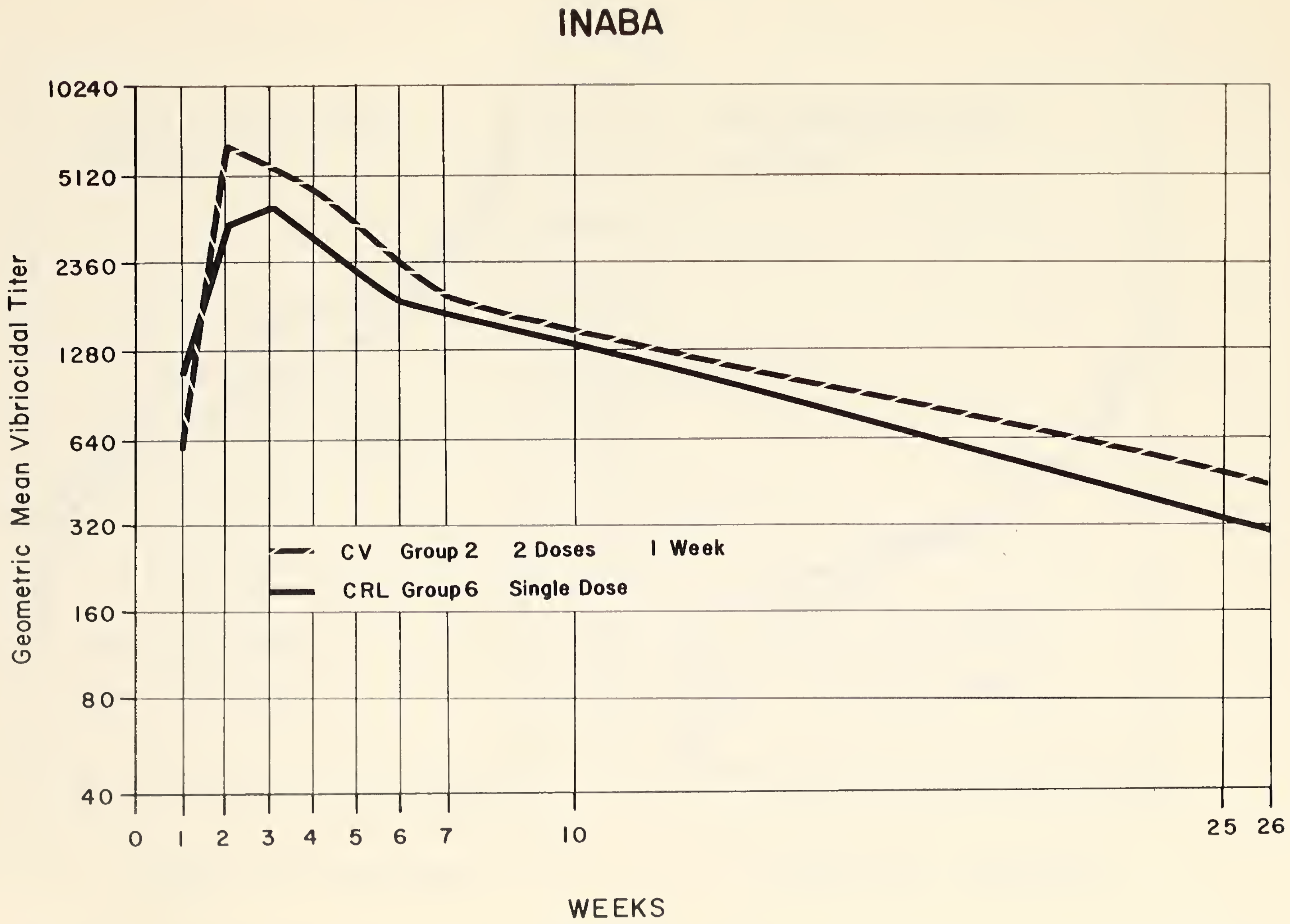

FIG. 7. A COMPARISON OF THE ANTI-INABA VIBRIOCIDAL RESPONSES OF VOLUNTEERS RECEIVING CRL AS A SINGLE DOSE OR CV AS 2 DOSES A WEEK APART

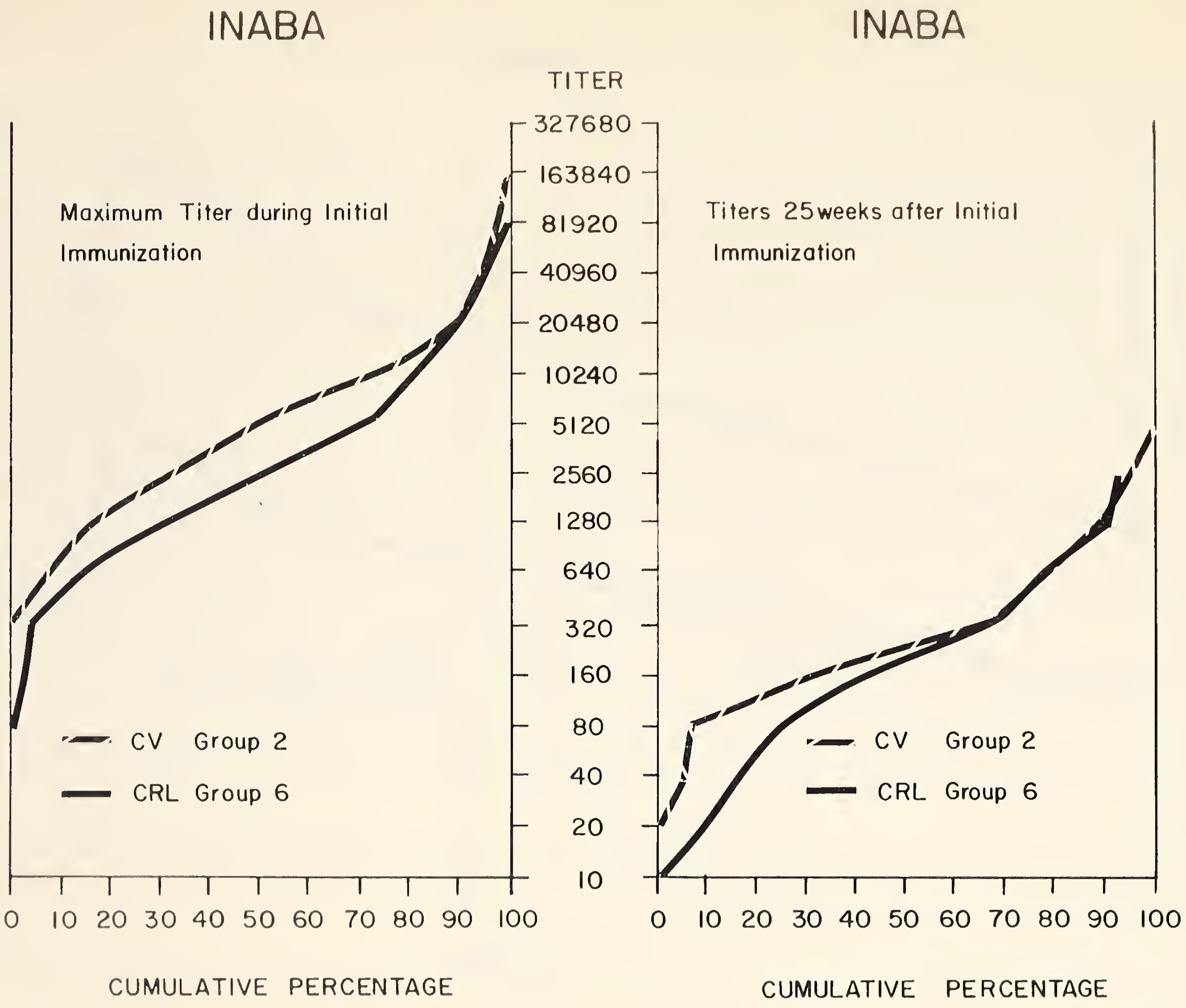

FIG. 8. CUMULATIVE PERCENTAGE DISTRIBUTION OF ANTI-INABA TITERS AT THE BEGINNING AND END OF THE INITIAL IMMUNIZATION PERIOD. THESE VOLUNTEERS RECEIVED CRL AS A SINGLE DOSE OR CV AS 2 DOSES A WEEK APART

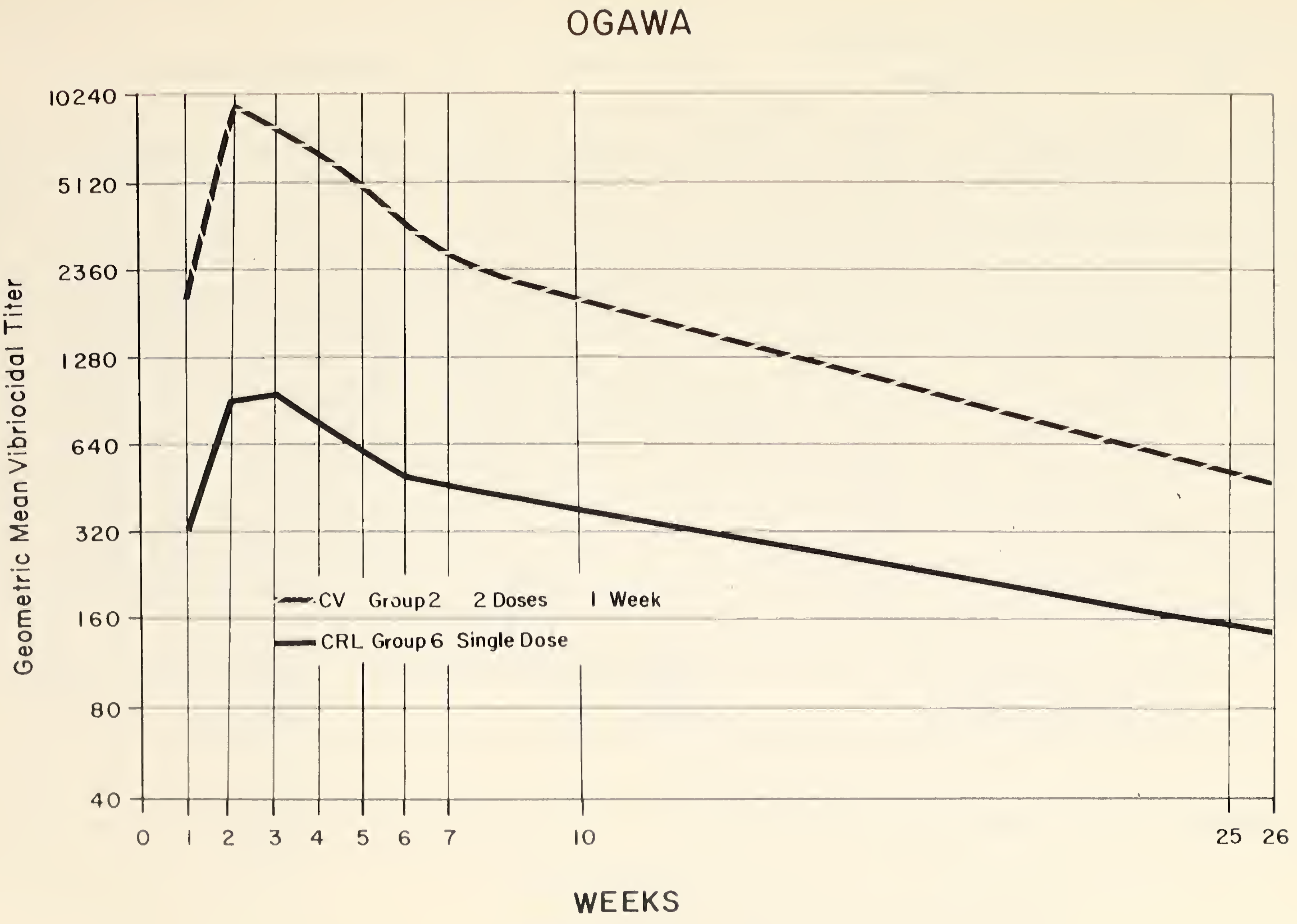

FIG. 9. A COMPARISON OF THE ANTI-OGAWA VIBRIOCIDAL RESPONSES OF VOLUNTEERS RECEIVING CRL AS A SINGLE DOSE OR CV AS 2 DOSES A WEEK APART

		Initial Immunization			Re-Immunization			Maximum Geometric Mean Vibriocidal Titer			
								Ogawa		Inaba	
Group	N	Antigen	Doses	Interval	Antigen	Doses	Interval	Initial	Re-Imm.	Initial	Re-Imm.
1A	17	CV	1		CV	1		11.9	10.8*	10.6	10.2
1B	16	CV	1		CV	2	1 wk	11.6	11.1	11.0	11.0
2	18	CV	2	1 wk	CV	1		13.0	11.3*	12.5	11.1*
3A	18	CV	2	4 wks	CV	1		11.7	10.1*	11.6	10.3*
3B	17	CV	2	4 wks	CV	2	1 wk	11.8	10.7*	11.8	10.9*
4	17	DCV	1		CV	1		10.3	9.8	8.8	10.1 **
5	15	DCV	2	4 wks	CV	1		10.4	10.0	9.6	9.3
6A	18	CRL	1		CRL	1		10.4	11.4**	12.0	12.5

Reference Tube 8 = 1: 320
9 = 1:640
10 = 1:1280

Reference Tube 11 = 1:2560
12 = 1:5120
13 - 1:10240

* Significant decrease

** Significant increase

TABLE 5. Maximum geometric mean vibriocidal titers (regardless of week of occurrence) after initial immunization and re-immunization

(Tube numbers of the standard dilution series)

Group	N	Vaccine	Ogawa		Inaba	
			Initial	Re-Imm.	Initial	Re-Imm.
1A	17	CV	1.7	1.5[*]	2.0	1.6
3A[**]	18	CV	2.0	1.2	2.0	1.2
6A	18	CRL	2.0	1.1	2.3	1.5

[*] Not significant

[**] Since Group 3A received two initial immunizing doses 4 weeks apart the time of the maximum titer after the first dose was used in this calculation

TABLE 6. Average time (in weeks) required for persons in various groups to attain their maximum titers after one initial immunizing dose and after one re-immunizing dose of the same antigen

		Initial Immunization			Re-Immunization			Minimum Geometric Mean Vibriocidal Titer			
								Ogawa		Inaba	
Group	N	Antigen	Doses	Interval	Antigen	Doses	Interval	Initial	Re-Imm.	Initial	Re-Imm.
1A	13	CV	1	--	CV	1	--	8.3	8.5	7.0	7.5
1B	10	CV	1	--	CV	2	1 wk	7.1	8.8*	7.0	7.9
2	14	CV	2	1 wk	CV	1	--	8.9	9.5*	8.7	8.9
3A	10	CV	2	4 wks	CV	1	--	9.5	9.6	8.3	8.5
3B	10	CV	2	4 wks	CV	2	1 wk	8.5	9.2*	8.8	9.1
4	9	DCV	1	--	CV	1	--	7.1	8.2*	5.4	6.4*
5	11	DCV	2	4 wks	CV	1	--	6.6	8.4*	5.3	6.1
6A	12	CRL	1	--	CRL	1	--	6.6	8.4*	7.7	9.5*
6B	12	CRL	1	--	--	--	--	6.9	6.9	8.2	8.1

Reference Tube 5 = 1:40
6 = 1:80
7 = 1:160
8 = 1:320
9 = 1:640

* Significant increase

TABLE 7. Minimum geometric mean vibriocidal titers 25 weeks after initial immunization and 26 weeks after re-immunization

(Tube numbers of standard dilution series)

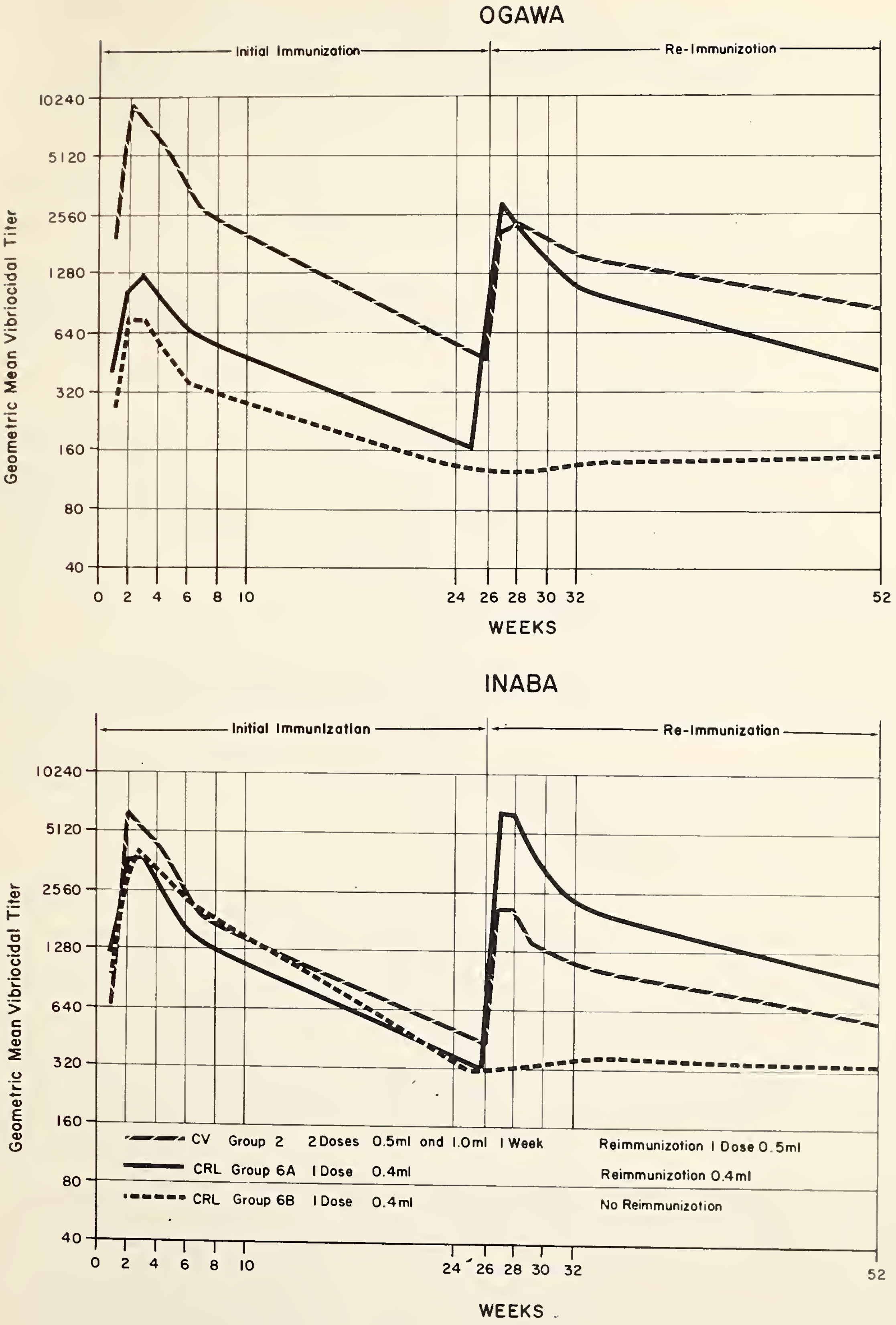

FIG. 10. MEAN VIBRIOCIDAL TITERS CALCULATED FOR PERSONS IN GROUPS 2, 6A AND 6B WHO COMPLETED THE FULL YEAR OF THE STUDY

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13 ABSTRACT

Human volunteers have been employed in the study of vibriocidal antibody responses to cholera vaccines administered by various regimes. Three vaccines of widely different potencies were used, the most potent being the one used in the Pakistan field trials of 1963-64. The regime producing the best overall response on initial immunization was the use of commercial vaccine of "normal" potency in doses of 0.5 ml and 1.0 ml 1 week apart. Two doses of this vaccine 4 weeks apart produced no evidence of a booster effect after the second dose, and the very high potency vaccine given as a single dose was not significantly better than two doses of commercial vaccine a week apart in regard to Inaba response, and definitely inferior in its Ogawa response. (U)

Re-immunization at 26 weeks produced group mean peak titers that were no higher and usually were lower than peak titers observed on initial immunization. No booster effect was apparent. Small-dose immunization followed by normal dose re-immunization had no advantages and double dose re-immunization was not significantly better than single dose re-immunization. The one group receiving only a single dose of vaccine maintained a constant and elevated mean titer from the 25th week to the 52nd week in the absence of any identifiable antigenic stimulation. (U)

Forty-seven percent of the volunteers in this study had vibriocidal antibody prior to immunization with at least 32% having no histories suggesting contact with cholera antigens. The occurrence of these natural antibodies was associated with improved responses to cholera vaccines. (U)

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Cholera vaccine						
Immunity						
Serological response						
Human						
Initial immunization						
Re-Immunization						
Dosage						
Interval						
Natural Immunity						

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